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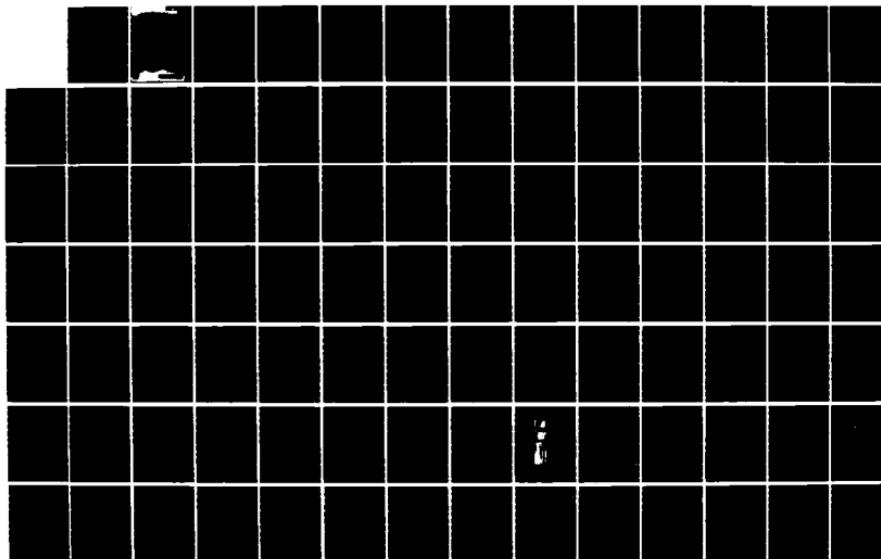
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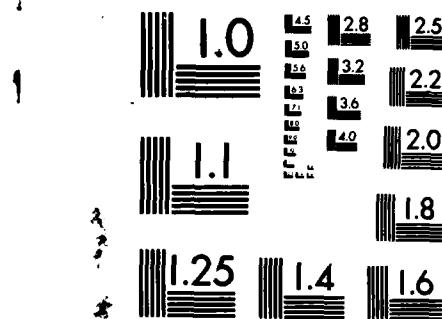
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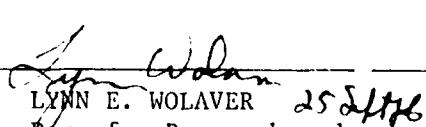
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EXTRACELLULAR ADENOSINE TRIPHOSPHATE
ASSOCIATED WITH AMPHIBIAN ERYTHROCYTES:
INHIBITION OF ATP RELEASE BY
ANION CHANNEL BLOCKERS

JAMES P. DIXON, B.S., M.S.

A Digest Presented to the Faculty of the Graduate School
of Saint Louis University in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

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Previous investigation has demonstrated that adenosine 5'-triphosphate (ATP) appears in the vascular effluent following skeletal and cardiac muscle contraction. By cannulating the frog heart and perfusing through it hypoxic Ringer's solution, this author observed elevated levels of ATP. The presence of blood elements, however, made it necessary to ascertain if the frog red blood cells (RBC's) were a source of ATP.

It was noted that RBC's up to $150/\mu\text{l}$ were present in the ~~clear~~ effluent samples of the frog heart. To study cell suspensions diluted to this level, whole blood was diluted with frog Ringer's solution. ATP was assayed by firefly lantern extract + D-luciferin (threshold 1 nM). Well-mixed suspensions produced ATP concentrations in proportion to the number of cells present. When cells were allowed to settle for one hour, nanomolar ATP concentrations in the supernatant fluid were observed, regardless of cell count.

Stirred cells did not increase the concentration of ATP in the medium, whereas cells allowed to settle in layers showed enhanced release of ATP. Anion channel blockers, probenecid and furosemide, diminished this release. Evidence that cell lysis was not the source of the ATP was based on two observations: 1) time course of the recordings of the "light signals" were identical for cell samples and ATP standards, and 2) digitonin lysed the cells in the assay solution, verifying their presence

after the assay. It is concluded that ATP is intimately associated with the external plasma membrane of this nucleated cell and the red cell must be considered as an ATP source in any sample where RBC's are present. It is hypothesized that ATP release occurs through an anion channel as a result of localized hypoxia.

Release of ATP from red cells has several implications. That ATP, a potent vasodilator, may be released because of localized hypoxia at rest indicates that the myogenic and neurogenic components in vascular tone are probably superceded by humoral factors during transient blood movement through the microcirculation. For example, ATP-stimulated release of endothelium-dependent releasing factor (EDRF) and subsequent vasodilation may occur routinely as blood moves from the arteries to the veins. Also, the nucleated red cell may serve as a model for cell-to-cell communication. The existence of ecto- enzymes, specifically ATPases, in the plasma membrane allowing control over the ATP that is released, may be important to physiological processes not unlike the cell differentiation that occurs during pulsatile release of cAMP from Dictyostelium discoideum. Finally, this nucleated cell may function as a model for the study of a rather unique anion channel that translocates a nucleotide.

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COMMITTEE IN CHARGE OF DISSERTATION

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INTRODUCTION

Interest in the vasodilatory properties of adenosine 5'-triphosphate (ATP) originated with Barcroft and Dixon (21). Forty years later two extensive works concerning extracellular ATP and its effects were published (141,177). Yet, only recently has the extracellular role of ATP received acceptance. Following the writing of the Historical Review, a review by John Gordon (171) was published on extracellular ATP that in many ways covered the same topics. Curiously, however, only minor duplication of the references is found, a striking indication of the vast literature on extracellular ATP.

One of the potential sources of ATP in the plasma is the blood elements. M.J. Rovetto in a recent review (368) aptly pointed out that red cell contamination may not be a trivial source of ATP, and indeed, that theme is a predominant conclusion of this dissertation. Included, of course, is documentation of the conditions under which ATP was associated with nucleated red blood cells, and why lysis was not the reason for the appearance of ATP.

These observations on red blood cells occurred during the study of ATP release from the hypoxic frog heart. It led to efforts to try to discover why ATP seemed to be released by red cells, and ways to control it. Additional research provided results on intracellular ATP levels in these cells and the ability of intact red cells to hydrolyze ATP.

An extensive review of the literature is provided, with major emphases on purinergic neurotransmission, purinoceptors, ATP vasodilation, endothelium-dependent vasodilation, and ATP release mechanisms. It provides a basis for describing the pervasiveness and potency of ATP and why it is a major concern in vasodilation, inotropic influences in the heart, and in the control of oxygen delivery in red cells.

The research results are the first describing and quantifying ATP release across a cell membrane via an anion channel. The evidence, however, is incomplete. The exact cause of ATP release is not known with certainty to be hypoxia, although the rationale is strong. The exact mechanism for ATP release is not known with certainty to be a channel, except that the evidence points in that direction. The reader should find extensive food for thought and a multitude of ideas for laboratory work can be gleaned from this information. Foremost, one should not come away from this work without an appreciation of how little is known about cell membranes and nucleotide release mechanisms. The way in which ATP is utilized other than as a source of energy has opened up a novel field of investigation and may be of great biological significance.

HISTORICAL REVIEW

Adenosine 5'-triphosphate (ATP) has a multiplicity of roles. Within the intracellular compartment, it functions primarily as a source of energy for various metabolic reactions. In this capacity ATP is typically described as a high-energy compound, i.e., a compound with a bond that when hydrolyzed yields a large, negative free energy change. Energy is conserved for use in anabolic processes within the cell when ATP is produced during the catabolism of nutrients. This intracellular role, however, is not the only function of ATP. Yet, the ATP molecule is usually discussed from this "energy" perspective.

It is, therefore, understandable why many biomedical scientists have previously thought that the release of ATP to the extracellular milieu was a loss of energy. Why would cells release a high energy compound to the outside of any cell? It would only be hydrolyzed by extracellular enzymes and eventually be excreted as uric acid. Additionally, if an extracellular function could be identified for ATP, how could this charged, anionic molecule cross the plasmalemma?

Presently, extracellular roles for ATP cannot be disputed on the basis of conservation of energy. The whole topic of cell-to-cell communication recognizes diversity of mechanism, not necessarily economy of action. For example, various peptides, which are more biochemically expensive to make than ATP, are now

known to have numerous extracellular roles and even to fulfill many of the criteria for neurotransmitter classification (see below). ATP release from excitable tissues, particularly nerves, has been demonstrated (2,271), and indeed, a neurotransmitter role has recently been attributed to ATP (57).

Another major, though controversial role for ATP is as a local metabolite in producing vasodilation. This role has its roots in the original work by Forrester (142,143), who showed that ATP exists in higher concentration in the plasma following muscle contraction.

Besides these two roles, other documented effects that ATP has on cells will be reviewed. Additionally, the following issues will be addressed:

- 1) Is ATP, or a metabolite such as adenosine 5'-disphosphate (ADP), adenosine 5'-monophosphate (AMP), or adenosine (ADO) the cause of the observable event? Vis-a-vis functional hyperemia, what evidential arguments can be made for and against the "adenosine hypothesis" and the "ATP hypothesis"?
- 2) What is the source of the extracellular pool of ATP?
- 3) What is the "signal" which causes ATP release from a cell?
- 4) What is the manner by which ATP departs the intracellular space?
- 5) What receptors on which cells are the means by which purine nucleosides and nucleotides cause an effect?
- 6) What enzymes in the extracellular environment are needed to maintain ATP at concentrations which are physiological?

ATP As a Neurotransmitter

Putative neurotransmitters must be shown to exist in and be released from the depolarized nerve terminal, have a source of enzymatic synthesis and of removal (degradation and/or uptake) from the synaptic cleft, be mimicked by exogenous application of the substance, and be blocked by known antagonists of the putative transmitter (60). ATP has not been shown to satisfy all the aforementioned criteria in a given tissue. For this reason, the following evidence is technically incomplete in supporting the neurotransmitter role for ATP.

Exemplary of the problems encountered in studying ATP as a neurotransmitter is the fact that more evidence is required than just showing that some purines appear after prolonged depolarization of excitable tissues (2,458,459). The exact substance and its origin must be ascertained. For example, Israel and co-workers (227,228,296) demonstrated using a firefly lantern bioassay method specific for ATP that nerve stimulation in Torpedo marmorata caused release of ATP. The origin of the ATP was shown to be the post-synaptic membrane of the electroplaque because its release was separate from that of acetylcholine (ACh). Curare, a post-synaptic nicotinic receptor blocker, significantly reduced ATP release, and carbachol (an ACh agonist) was shown to elicit ATP release. It was suggested that ATP released from the post-synaptic membrane modulated the pre-synaptic release of ACh.

Israel's work also provided strong evidence that depolarization is one method for ATP release from a membrane. Forrester (146) argues accordingly: depolarization of an excitable membrane is the earliest possible signal (biophysical signal) for providing a fast response. White's research (458,459), utilizing the same firefly technique, demonstrated ATP release during depolarization of synaptosomes, which lends additional support for this hypothesis. Important to this research was showing that tetrodotoxin, a sodium channel blocker, blocked the release of ATP caused by veratridine. Thus, membrane depolarization was required for ATP release.

Others (232,427) have also concluded that an appreciable amount of the labelled purines released from the cerebral cortex appears as ATP, though some groups demonstrated no specificity for these purines (102,352,353). Without specificity, one does not know if adenosine is the substance predominantly released or whether in fact it may have been ATP (379,427). White's technique (458) overcomes this obstacle.

The research of Israel and co-workers demonstrating ATP release in the Torpedo exemplifies a neuromodulator role, rather than a transmitter role, for ATP (see also 8,240,307). An example of pre-synaptic release of ATP, and therefore evidence for a possible transmitter role, is the rat phrenic nerve-hemidiaphragm

preparation. Silinsky (395,397) demonstrated that with curare present at a concentration sufficient to suppress end-plate potentials nerve stimulation still resulted in the appearance of ATP and ADP. Concentrations of ATP up to 0.1 mM in the synaptic cleft were estimated. If carbachol was present, no nucleotide appeared. Treatment with hemicholinium-3 to block choline re-uptake and deplete the vesicles, also caused a decrease in nucleotide release. Silinsky concluded that ATP release was pre-synaptic.

This work is also an early example of co-transmission of ATP and ACh, i.e., multiple neurotransmitter release from the same vesicle. Co-transmission (22,60,242,359) was recently examined vis-a-vis Dale's Law (324), and found to be the rule rather than the exception. Silinsky's work (395) showed that as nerve-stimulated ACh release declined in the presence of low calcium (used to inhibit vesicle exocytosis), nucleotide release also declined. Evidence by others (360) indicated that ATP probably decreases the amplitude of the end-plate potential, and thereby inhibits excitation. This hypothesis is not supported by other research, but rather suggests a facilitatory action by ATP on the post-synaptic membrane (134).

The most complete evidence in one tissue for ATP functioning as a co-transmitter comes from the study of the vas deferens in the guinea-pig (294,403,404,415,416), mouse (415,416), and rat (153). To summarize Sneddon & Westfall's work in the guinea-pig

(404), it was shown that

- 1) tetanic nerve stimulation in the guinea-pig vas deferens resulted in a biphasic response:
 - a) a rapid, phasic contraction, and
 - b) a slower, tonic contraction.
- 2) the initial phase of contraction was mimicked by ATP and β, γ -methylene ATP.
- 3) the second phase was mimicked by norepinephrine (NE).
- 4) arylazidoaminopropionyl-ATP (ANAPP₃), a P₂ receptor antagonist, blocked the initial phase of contraction during stimulation and application of ATP.
- 5) prazosin, an α_1 -receptor antagonist, blocked the second phase during stimulation and during application of NE.
- 6) yohimbine, an α_2 -receptor antagonist, enhanced both phases (by preventing pre-synaptic feedback).
- 7) reserpine, which depletes catecholamine stores (81), reduced the second phase; excitatory junction potentials (e.j.p.'s) recorded with an intracellular electrode were unaffected by reserpine.
- 8) the e.j.p. could be mimicked by ATP but not by NE.
- 9) ANAPP₃ reduced the e.j.p. without changing threshold or the ability to fire an action potential.
- 10) cocaine (an NE uptake blocker) affected tension development by enhancing the tonic phase of contraction and depressing the phasic contraction.

Additionally, Westfall (456) showed that ATP blocked NE release, ANAPP₃ antagonized the latter effect, and phentolamine (an α_2 -antagonist) increased NE release. He concluded that 1) ATP and NE are co-transmitters in this tissue; 2) that ATP acts via a P₂ receptor to cause e.j.p.'s post-synaptically; 3) that e.j.p.'s can summate to cause an action potential, resulting in a phasic contraction; and 4) that ATP acts via a pre-synaptic P₂ receptor to inhibit transmitter release.

It has recently been proposed (137) that identification of this co-transmitter function of NE with ATP may explain an

unresolved paradox. The e.j.p.'s in this preparation are insensitive to α_1 -antagonists, but can be blocked with ANAPP₃. Thus, it is unlikely that two populations of excitatory adrenoceptors exist (99), such that the "intrag�unctional" (γ -adrenoceptor) is resistant to α and β blockers, and the "extra-junctional" adrenoceptor is not. When adrenergic antagonists do not work, purinergic influences may be suspected.

There is other, though less complete, evidence for ATP functioning as a neurotransmitter (344,420). Among the various modulator or transmitter roles that it serves, ATP has been shown to provide feedback inhibition of NE (152,332,425) and ACh (396) release as well as function as a primary or as a co-transmitter. ATP may function as a possible neurotransmitter in sympathetic innervation of cardiac muscle (350), as a co-transmitter in the adrenal medulla (121,122,363), and as a transmitter in amphibian sympathetic ganglion to inhibit the M current (8). ATP may affect dorsal root terminals in the toad spinal cord (343), and function as a CNS neurotransmitter for mechanoreceptor afferents synapsing in the substantia gelatinosa and nucleus proprius of rat and cat (117,163,230). ATP has also been shown to excite free sensory nerve endings in the skin (40).

Smooth muscle is also influenced by purinergic input.

Accordingly, Burnstock (62) attributes to both ATP and adenosine a role as primitive transmitters. ATP may mediate contraction in the urinary bladder of the rat and guinea-pig (53,63,99,238), relaxation in taenia coli (17,63,87,173,380,381) and relaxation of the longitudinal and circular muscle of the ileum via the myenteric plexus (11,240,383,450,475). ATP may also function as the non-cholinergic, non-adrenergic innervation in the chicken and lizard rectum (295,382) and the rabbit rectococcyeus muscle (88,405).

Evidence is also abundant for ATP functioning as a neurotransmitter in blood vessels (59,62,243,244,245,286, 308,402,423,424,425,426), producing relaxation in some and contraction in others. However, more information is required concerning the location of purinergic receptors in resistance vessels (62). Though it is frequently thought that ATP causes vasodilation only via the endothelium-dependent relaxing factor (EDRF) of endothelial cells (62,157) (see p. 23), perfusion of ATP solutions through specific blood vessels without endothelium can cause relaxation (148,156,159), albeit less than endothelium-dependent relaxation (111,156,159). EDRF-relaxation by ATP is a topic that is further addressed in a later section.

The evidence for transmitter action has been reinforced by

patch-clamp studies. Kolb and Wakelam (260) characterized ATP-sensitive cation channels in embryonic skeletal muscle. Krishtal et al. (268) also demonstrated cation-specific conductance changes produced by ATP in micromolar concentrations in isolated neurons from various sensory ganglia, particularly the vestibular ganglia. Clarification of these purinergic roles in the control of membrane channels can be expected as electrophysiological and pharmacological techniques are applied (195,236,241,322) and the specific mechanisms of ATP action on membrane receptors is delineated.

There are several observations of clinical importance to neurology which deserve mention. Hansen (190) presents data that patients diagnosed as having schizophrenia, or psychotic/neurotic depression have elevated blood levels of ATP and/or adenosine. Also, caffeine addiction involving purinoceptors has been clinically associated with schizophrenia and anxiety-provoking/depressant symptoms in psychiatric patients (176,298). This suggests that some unknown, abnormal purinergic mechanisms may be involved.

The same comment may be made concerning abnormalities of the extrapyramidal motor system, for which ATP administration is sometimes combined with neuroleptic drug therapy to alleviate some symptoms (109). Basal ganglia disorders may account for dyskinesias and biting tendencies in the Lesch-Nyhan syndrome, whose primary defect involves a lack of the purine salvage enzyme,

hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) (235), an enzyme found in basal ganglia nerve terminals. If high blood purine levels are indicative of extracellular levels, then normal function of extrapyramidal neurons may not be possible if HGPRTase activity is decreased or absent.

Muscular dystrophy is another clinical disorder for which abnormal purine metabolism may be important. For instance, there may be reduced ATP availability because purine phosphorylation in cells is diminished (406,432), a finding consistent with the decreased deformability and increased fragility of erythrocytes in Duchenne patients (170) and during red cell ageing and nucleotide depletion (284,309,310). There is also an increase in the amount of 5'-nucleotidase (25) and possibly HGPRTase (314) in dystrophic skeletal muscle. Allopurinol, a xanthine oxidase inhibitor, has proved to be of some benefit to these patients (432). Stone (419) reviews the interesting hypothesis that a vascular defect may underlie muscular dysfunction in this disease. Especially concerning the ATP hypothesis of vasodilation (see below), it is important to emphasize that patients with muscular dystrophy exhibit reduced vascular blood flow in exercising muscle (419).

Purinoceptors

The primary reason for distinguishing the purinoceptors into two types, P_1 and P_2 , was originally based on the selective actions of their agonists and antagonists (58). At that time, methylxanthines were known to block adenosine actions. The order

of agonist potency for P_1 receptors placed adenosine first, followed by AMP, ADP, and ATP. Increased 3',5'-cyclic adenosine monophosphate (cAMP) was thought to result from P_1 stimulation. P_2 receptors, on the other hand, caused no cAMP changes and were said to be activated best by ATP with successively less agonist potency displayed by ADP, AMP, and adenosine. P_2 receptors were antagonized by 2'-2-pyridilisatogens, 2-substituted imidazolines (for example, antazoline and phentolamine), and quinidine, albeit with variable effectiveness. The antagonism for P_2 purinoceptors was, therefore, non-specific.

Differentiation between the major purinoceptors has now been expanded to discriminate between subtypes of each (64,171). Adenosine receptors (P_1) are of two types. The A_2 (or R_a) receptor stimulates adenylate cyclase, and the A_1 (or R_i) inhibits adenylate cyclase (342). Both function via GTP-dependent mechanisms, with the A_2 action being mediated via a G_s -protein subunit, and A_1 action via a G_i -protein subunit (101). Methylxanthines such as caffeine, theophylline, and isobutylmethylxanthine (IBMX) remain effective antagonists for both adenosine subtypes. Clonidine has also been shown to be a non-specific antagonist in several selective tissues (241). Further advancement in adenosine-receptor mechanisms can be expected due to efforts to develop radioactive adenosine analogs (101) and to the recent discovery of naturally occurring analogs from marine organisms (104).

Experience with the ATP receptors (P_2) has shown them to have multiplicity of action, also. The criteria for differentiating the subtypes is still based on agonist potency and antagonist activity. There are two subtypes (64,171). P_{2X} receptor subtypes are more responsive to α, β -methylene ATP, followed by β, γ -methylene ATP, ATP, and 2-methylthio-ATP. Arylozido-aminopropionyl-ATP (ANAPP₃) is a photo-activated, irreversible antagonist of this P_2 subtype (241). A good example of its inhibitory effects is in guinea-pig vas deferens (138,404). P_{2X} receptors usually mediate excitation of both smooth and cardiac muscle.

The other subtype, P_{2Y} , is weakly antagonized by ANAPP₃ and mediates smooth muscle relaxation directly or by causing the release of endothelium-derived relaxing factor (EDRF) or prostacyclin. Good examples of P_{2Y} -mediated relaxation are seen in rat aorta (460) and in rat femoral artery (245). The order of agonist potency is 2-methylthio-ATP, ATP, α, β -methylene ATP, and β, γ -methylene ATP.

This stereoselectivity of P_2 receptors exists in direct contrast to the stereospecificity of P_1 receptors for adenosine. It also contrasts with the ADP-specific purinoceptor on platelets (197,301). Both P_1 and P_2 receptors require:

- a) NH₂ at the C6 of the imidazole ring, and
- b) the ribose moiety.

Thus, inosine (NH₂ absent) and adenine (ribose absent), or the respective phosphorylated congeners, are ineffective agonists (58).

It is necessarily worthwhile to be mindful of several facts concerning purinoceptors. First, their location is not always indicative of their mode of action. For example, some P₂ receptors located on smooth muscle may be excitatory or inhibitory (244,289). Thus, ATP may not need to release an EDRF to relax resistance vessels. Presently, the receptor population in resistance vessels is unknown. Second, Burnstock (32,33) is of the opinion that P₁ and P₂ receptors are located exclusively on the pre-synaptic and post-synaptic membranes, respectively. This has been shown otherwise (456). Third, apamin, a potent neurotoxin in bee venom, was once thought to be an ATP antagonist. It now is considered to function as a potassium channel blocker (60,64,138). Fourth, P₂ receptors may be more heterogeneous than currently portrayed. Besides P_{2X} and P_{2Y} and platelet purinoceptors, there are ATP receptors on rat mast cells responsible for histamine release (100,171). Finally, ANAPP₃ may not be specific for P₂ receptors. This uncertainty has been generated because of findings in rabbit anococcygeus muscle (405) and guinea-pig taenia coli (457), in which, as explained by Burnstock (64), ANAPP₃ may also antagonize responses to adenosine and AMP, which are considered to have a greater affinity for the P₁ receptor.

A Vasodilator Role for ATP

Movement of blood through capillary beds of muscle may be dependent on the type of muscle (223), but ultimately it is related to the level of oxygenation (P_0_2) of the surrounding tissue (257,278) and the need to remove metabolic substance(s) from the tissue (391). Both at rest and during muscle contraction, control over perfusion occurs as a result of neurogenic and metabolic contributions to vascular tone (391). Though the metabolic component is of greater importance during contraction, neither at rest or during exercise is it known by what method or at what location oxygen needs and requirements of waste removal are transduced to effect the release of a vasoactive metabolite. Without discussing the full complement of metabolites that are candidates for this vasodilatory substance, the following comments are intended to polarize the reader's thoughts by providing convincing evidence why ATP may be considered one of these metabolites.

Virtually the only extracellular fluid which has been examined for ATP levels is the plasma. It was Forrester (143) who applied the firefly bioassay technique of Strehler and McElroy (422) to show ATP in the plasma, providing one of the first definitive efforts that quantitatively demonstrated an increase in ATP in the effluent from exercising muscle. ATP, though, is not the only substance important in the control of vascular resistance (145,146,186,391,407,448).

Forrester (145) reviews the historical basis for his efforts and cites Barcroft and Dixon (21) for the earliest of statements placing purine compounds in a vasodilator capacity. He also cites Abood et al. (2) as the first to demonstrate ATP efflux from excitable tissue. Prior to Forrester's initial findings, however, there was only circumstantial evidence based on intravascular ATP infusions that endogenous ATP could be a potent vasodilator in peripheral (55,126,132,141,177) and coronary circulations (467,469). Since that time, ATP has been assayed in the venous effluent of skeletal muscle (42,46,142,144,147,149,150,192,464), working frog myocardium (124), working rat myocardium (82), in the supernatant fluid of isolated rat cardiocytes exposed to hypoxia (151), and in the effluent from hypoxic and acetylcholine-stimulated guinea-pig heart (326,389). This has generated controversy because rather than ATP, it is adenosine, an ATP metabolite that is a potent vasodilator, that is generally described as the metabolic substance that is released from muscle to cause exercise hyperemia. The reasons for this controversy are two-fold.

Though there is no disagreement that adenosine (an uncharged purinergic compound) can pass through the plasmalemma, it has generally been thought that adenosine does so after intracellular ATP has been dephosphorylated and depleted. Thus, a metabolite (adenosine) is released across a membrane to initiate vasodilation. This is the textbook explanation (183) for functional hyperemia in both skeletal and cardiac muscle, though

the hypothesis was originally based on research in coronary circulation (28,229,239,361,372). Some research efforts in skeletal muscle circulation (43,116,185,390) have since concluded that adenosine is also the peripheral vasodilator substance. The first reason for controversy then, is that research efforts have been overwhelming in demonstrating adenosine in the venous effluent. This is the foundation for the "adenosine hypothesis" (29,30).

The second reason for controversy surrounding the ATP hypothesis is that ATP has generally been considered to be impermeable to the plasmalemma, primarily because it is a charged molecule. There may have been an open-mindedness concerning passage of this molecule through the cell membrane prior to 1952, but the Nobel Prize-winning efforts of Hodgkin and Huxley (212) changed opinions about membranes. Forrester (145) attributes to the Hodgkin-Huxley membrane theory the demise of efforts to demonstrate a vasodilation role for ATP. Likewise, Chaudry (79) ascribes to Glynn (169) reasons for lack of acceptance that ATP moves across cell membranes. What reasons exist, then, for considering ATP as a vasodilator?

First, as cited above, ATP levels have been found to be elevated when hyperemia occurs in response to muscular contraction. One group (42) observed both adenosine and ATP in the effluent, but concluded that ATP had not been found, and so entitled their paper! In spite of such prejudice and attempts to

prove that ATP does not flow from contracting muscle (474), the ATP hypothesis cannot be ignored because it has been shown to be released at concentrations high enough to account for exercise hyperemia (150).

Elevated levels of ATP could originate from one or any combination of several tissues. Nerve terminals and varicosities, as discussed previously, may be a source as well as a target for ATP. Striated and smooth muscle cells, because of their high (millimolar) ATP concentration, may release ATP. Endothelial cells, which have been shown to release ATP (335,337,339), could be a major source, since they constitute a vast surface (1000 cm^2/gm heart vs. cardiomyocytes of $250-500 \text{ cm}^2/\text{gm}$ heart) and have a higher content of ATP (≈ 3 times) compared to myocardial cells (165,316). Finally, blood elements could be a source. Platelets have been shown to release both ATP and ADP when activated (44,90,224,263). Platelet activation and ATP release due to trauma are special situations not addressed in this review.

A second reason for considering ATP as a metabolic vasodilator is potency. A substance that causes vasodilation must be very potent in order to increase blood flow from $2-3 \text{ ml}/100 \text{ gm} \cdot \text{min}$ at rest to $250 \text{ ml}/100 \text{ gm} \cdot \text{min}$ (14). Though it is recognized that both purines, adenosine and ATP, are powerful vasodilators, ATP is without question more potent (126,177,305,469). Recent evidence (148) suggests ATP is able to cause relaxation of resistance arterioles at threshold concentrations (10^{-10}M) 1000 times less than adenosine. If the P_2 receptor recognizes

ATP⁴⁻, then even this potency estimate may be imprecise by orders of magnitude (98).

Refutation of the adenosine hypothesis is not difficult when the facts are known. The evidence is decisively against it. First, it is fundamentally important to ascertain if the putative vasodilator, in this instance adenosine, is present to act. Forrester (145) and Haddy and Scott (186) cite references that emphasize that in skeletal muscle cells, an intracellular adenylylate deaminase degrades AMP to inosine monophosphate (IMP), completely bypassing adenosine (225,433). This pathway is also characteristically observed in endothelial cells (336), and has been described in detail in the leukocyte model (317), and clinically in a case of adenylylate deaminase deficiency (376). Since IMP is not a vasodilator (469), it is only possible that the adenosine seen in the venous effluent of skeletal muscle (42,142,144,149) exists as the result of the extracellular hydrolysis of ATP. The topic of ecto-enzymes is considered later.

Adenosine formation only occurs intracellularly after serious impairment of energy metabolism during exercise. If there is considerable breakdown of ATP and ADP in the muscle cell, then 5'-nucleotidase is no longer inhibited and consequently removes the α -phosphate moiety from AMP. Based on data from the rat heart (19,280) showing a K_i of 1.8 μ M and 0.08 μ M for inhibition of 5'-nucleotidase by ATP and ADP, respectively, one may reasonably conclude that this enzyme is totally and continuously inhibited by

the millimolar concentrations of ATP found in muscle cells. Intracellular formation of adenosine is, therefore, highly improbable (204). Thus, another reason for accepting the ATP hypothesis is that adenosine is formed, not intracellularly, but from the enzymatic degradation of ATP by ecto-enzymes on the outside of cells.

Evidence exists that adenosine may be insufficient as the sole metabolic vasodilator. In coronary circulation, aminophylline or theophylline blocks the vasodilatory effects of exogenous adenosine (4,6,56,234,447) but not ATP- (166) or hypoxia-induced vasodilation (5,234). Aminophylline and theophylline have been shown to antagonize adenosine-induced vasodilation in skeletal muscle (222,429) but not exercise hyperemia or hypoxia-induced vasodilation (306). Perfusion with high concentrations of adenosine into skeletal muscle to saturate P_1 receptors fails to prevent both functional and reactive hyperemia (208). Though this was not observed in coronary circulation (95), perfusion with adenosine deaminase did not decrease coronary flow (269), indicating that adenosine alone is insufficient as the sole metabolic vasodilator.

Evidence with antagonists is not always clear. For example, Forrester (145) emphasizes that studies utilizing dipyridamole, an adenosine uptake blocker, are inconclusive because this drug can also potentiate ATP action, and consequently prevent discrimination between the actions of these two purinergic substances. Nevertheless, a fourth reason for accepting the ATP hypothesis is

the fact that adenosine antagonists do not block functional hyperemia. Consequently, adenosine cannot be the sole vasodilating substance.

In this same context, it is highly unlikely that enough adenosine is formed intracellularly to cause vasodilation in the course of one brief contraction. Such a contraction is all that was needed to cause a decrease in resistance during perfusion of dog gracilis muscle (221) and an increase in blood flow in human forearm muscles (93). In the gracilis muscle, it was postulated by Honig and Frierson (221) and Honig (220) that neurons intrinsic to the vasculature exist that are responsible for such fast hyperemia. They presented convincing evidence that vasodilation occurred quickly following a single twitch. They further demonstrated that theophylline did not alter the rate of formation or the magnitude of dilation, but almost completely abolished post-exercise hyperemia (222).

A related phenomenon, called a "defense reaction" after the behavioral activity of the cat, has been demonstrated in rabbits. Shimada and Stitt (393) have shown that electrical stimulation of the hypothalamus elicits a neurogenic vasodilation independent of muscle activity. Antazoline (a non-specific P₂ antagonist) attenuated this dilation, but aminophylline had no effect.

One approach that initially seemed convincing for the adenosine hypothesis involved the infusion of venous blood from a contracting skeletal muscle into a resting skeletal muscle or a kidney. Blood vessels were dilated in the recipient resting

muscle and constricted in the kidney (390). Since ATP dilates renal vessels but adenosine constricts (196,390,436), one may logically conclude that adenosine was in the effluent from the exercising muscle. This does not rebut the ATP hypothesis, however. ATP should not be expected to last very long in whole blood because of the high ATPase activity associated with red blood cells (see below). If ATP were responsible for hyperemia in the exercising muscle, there is no reason to expect that it would remain at high concentration once it entered the blood. Adenosine, though, should be expected from the extracellular and intravascular hydrolysis of ATP.

When ATP appears in the blood from whatever source, there is strong reason to believe that any existing vasodilation, whether of neurogenic or metabolic origin, would be reinforced by the effect of ATP on the endothelium. This phenomenon is called endothelium-dependent vasodilation and is due to the release of an unknown substance called endothelium-dependent relaxing factor (EDRF).

The Role of the Endothelium in Vascular Smooth Muscle Responses to Purines

Prior to Furchtgott and Zawadski's accidental discovery of endothelium-dependent vasodilation (158,160,162), the basis for vascular dilation was thought to be the result of the influence of a metabolite or transmitter directly on the smooth muscle of the blood vessel. The number of vasodilatory candidates was and

still is high (391,407). Once the endothelium was found to communicate the presence of vascular constituents, the scenario became more complicated. Infused substances (e.g., acetylcholine) that caused vasodilation when the endothelium was intact exhibited a contractile influence or no influence at all when the endothelium was removed by rubbing or collagenase treatment (157).

The degree of smooth muscle contraction, or vascular tone, has been shown to be important in demonstrating endothelium-dependent vasodilation. This may be because EDRF is being continually released (181,299). Pre-contraction with norepinephrine or a prostaglandin (PGF_{2 α}) is usually the procedure for demonstrating EDRF effects (162,291,371). The in vivo response of vascular smooth muscle will consequently depend on the presence or absence of the endothelium, and the concentration and location (luminal or abluminal surface) of other substances that affect either the smooth muscles, the endothelium, or both.

In the presence of ATP, vasodilation results (111,172). P₂ purinoceptors are located on endothelial cells (203,243), and mediate the release of an EDRF when ATP or ADP is present (172,356). ATP also can cause transient release of prostacyclin (PGI₂) from endothelial cells in some vascular beds (171,203,291,333,340). Under these circumstances ATP, if present, causes vasodilation via PGI₂ effects on smooth muscle (333). Thus, ATP can cause endothelium-dependent vasodilation via EDRF or PGI₂, by acting from the luminal side of the blood vessel on an alleged P_{2Y} receptor (171).

Endothelium-dependent relaxation of smooth muscle may be additive to the vasodilation seen during exercise. When the ATP gradient from the interstitial space to the vascular space increases, endothelium-dependent mechanisms are invoked. It also is possible that endothelium-dependent vasodilation may occur routinely as blood flows through the circulation and blood elements interact with the endothelium.

The EDRF that is released by ATP is presently unknown (86,156,161,440). The candidates for EDRF have been most recently described as epoxides, aldehydes, lactones, lipoperoxides (107,180), or platelet activating factor (PAF) or atrial natriuretic factor (ANF) (333). Other evidence (421) refutes that PAF is an EDRF. EDRF does not evolve from the cyclooxygenase or lipoxygenase pathways (180,252) and is probably not a free radical (180). The mechanism of EDRF release requires calcium (279,161), but little else is known about it other than it has a short half-life in seconds (65). Once the EDRF is released, the smooth muscle is caused to relax by a mechanism that involves the elevation of cyclic 3',5'-guanosine monophosphate (cGMP) activation of a cGMP-dependent protein kinase, and dephosphorylation of myosin light chain kinase (113,125,216,255,256).

Endothelium-independent relaxation by purines involve P₁ or P₂ purinoceptors on the smooth muscle. P₁ receptors are known to exist on smooth muscle cells, and are generally considered to be the means by which adenosine causes relaxation. Recent

evidence also argues for vasodilation via P_1 receptors on the endothelium when adenosine is in the blood (315).

Direct vasodilatory action by ATP on smooth muscles is sometimes discounted because P_2 receptors are thought to be sparse on smooth muscle membranes (159). Though the predominant evidence is that ATP vasodilation is EDRF-mediated (110,111,112,156,162,172,243,245,356,441,460), it should be emphasized that in vivo conditions may be different. All of the experimental protocols studying endothelium have utilized isolated, large blood vessels. Resistance arterioles in vivo may function differently depending on the receptor population, an observation already made with adenosine responsiveness (374). Other evidence also suggests that the pharmacology of resistance vessels is not comparable to conduit vessels (362).

P_2 receptors also exist on vascular nerves and may suppress norepinephrine release from sympathetic vascular nerves (392,444). This may be one of the ways in which ATP from an abluminal source contributes to functional hyperemia.

The evidence seems to indicate that adenosine does appear in venous effluent (185), but only after long-term skeletal muscle exercise and certainly not in the early seconds of contraction (43,116,187). A contribution by adenosine to sustained exercise hyperemia (221), to post-exercise hyperemia (222,248,429), and to reactive hyperemia (305,436), have been proposed. Again, the evidence shows that the source of this adenosine is the breakdown of extracellular ATP unless the metabolism of the muscle cell has

been sufficiently compromised to override the mechanisms that exist for replenishing ATP: phosphocreatine, glycolysis, myokinase reaction ($2 \text{ ADP} \rightarrow \text{ATP} + \text{AMP}$), and oxidative phosphorylation. Only then might adenosine form a gradient out of the cell.

Evidence for the metabolic component in exercise hyperemia appears to support the ATP hypothesis. If intrinsic neurons contribute to functional hyperemia (218,219), it may be ATP from these neurons [in accordance with the research of Shimada and Stitt (393)] that is the operative agent. A strong case exists for postulating both neurogenic (220,221,393) and metabolic (145) components for ATP vasodilation. Either way, a mechanism must exist for the ATP to be released.

ATP Release Mechanisms and Membrane Permeability

If the source of ATP is a nerve terminal or varicosity, then ATP may be released as a neuromodulator or transmitter in the classical exocytotic fashion. Release of ATP may also occur in a non-classical way, i.e., other than via exocytosis. In isolated vesicles from Torpedo, both acetylcholine and ATP concentrations diminished without a decrease in vesicle numbers (476). This may imply a neurotransmitter release mechanism other than exocytosis. Though exocytosis has recently been challenged, a discussion of quantal and non-quantal release is beyond the scope of this work. The reader is referred to some recent reviews (72,189,226,430, 446).

For the metabolic release of ATP to occur a trigger, or

signal, is imperative (146). Paddle and Burnstock (326), Williams and Forrester (463), Forrester and Williams (151) and Clemens and Forrester (82) provide evidence that hypoxia may be one signal that provides for the release of ATP from myocardium.

Depolarization of an excitable membrane is the earliest possible signal (biophysical signal) that may anticipate impending work demands. As already discussed, the results of Israel (227,228,296) and White (458,459) support this hypothesis. Forrester (146) draws attention to the fact that vasodilation occurs very rapidly and is not proportional to the strength of contraction, but rather to the impulse frequency of stimulation and the number of motor units recruited in a muscle (246).

The lipid barrier of the membrane should ordinarily prevent the passage of ATP. The question then arises as to whether or not an excitable membrane undergoes enough of a physical change during depolarization to allow the release of ATP. Given that the voltage change during membrane depolarization is due to the movements of sodium, potassium and calcium down electrochemical gradients and through ion selective channels, an electrochemical gradient for ATP must also exist due to the high concentration of negatively-charged ATP inside a cell. If the membrane were sufficiently perturbed, might not ATP exit the cell? Cohen et al.

(89) demonstrate action potential recordings and birefringence changes in an axon that are identical in shape and time, and for which they ascribe a physical change in the

membrane structure. Due to this physical alteration in the membrane, it can be hypothesized that for the time period of an action potential the membrane may not continue to function as a physical barrier to ATP.

ATP movement across a membrane by means of a translocator protein is one method of release for which there is experimental evidence. Carrier-mediated transport mechanisms have been described in adrenal chromaffin granules (1,261), synaptic vesicles from Torpedo electric organ (275,281,282), in cortical cells of rabbit kidney (290,453), and in rat hepatocytes (80). In each instance, the experimental evidence describes ATP influx. There are presently no known translocators for ATP efflux from a cell. The ADP/ATP counterexchange protein of the inner mitochondrial membrane has been characterized (16,41,133,254, 255,256), though it is technically an influx system, too.

Nucleoside transporters have been described in cultured cells (330,345,346,336), in erythrocytes (330,345,347) and in myocardial cells (369,465,466). Many inhibitors of the mechanism have been described. The erythrocyte transporter appears to have a broad substrate specificity and is inhibited by nitrobenzylthiopurine (NBTP) nucleosides (331). Studies of this transporter in other cells indicate that this protein is so integrally embedded in the membrane as to be unaffected by treatment with trypsin, neuraminidase, and other enzymes.

The adenosine transport system in cardiac myocytes provides

interesting new data concerning the loss and salvage of adenine derivatives in myocardium. Rovetto and co-workers have shown that the rate of adenosine uptake was two-fold greater from exogenous ATP (10 μ M), which first required dephosphorylation, than from exogenous adenosine (35 μ M) in cultured cells (466). This difference was not observed in the perfused heart, possibly because of ecto-enzyme activity (see below). Other evidence supports the hypothesis that adenosine uptake proceeds by two separate and distinct processes: one requiring ATP hydrolysis and the other free adenosine. These transport systems had K_m 's of 10^{-8} M and 10^{-5} M, respectively (369). The high affinity transporter, in response to a hypoxic insult, functioned in reverse by releasing adenosine from the cell. This process was inhibited by concanavalin A (465). It was hypothesized that, during hypoxia, free adenosine does not build up intracellularly because of this high affinity transport. Others describe adenosine diffusion across the plasmalemma down a concentration gradient (386).

Cultured astrocytes have been shown to release ATP (437). The author described this release as a translocation process which was sensitive to the concentration of ATP existing on the outside of the cell, with different cell lines responding differentially to various ATP concentrations. NN astrocytes, for example, responded to 5×10^{-7} M ATP, whereas others required a higher concentration and some cells (sea urchin, human erythrocytes and human oligodendrogloma cells) did not release ATP.

Some authors (205,370,454) have discounted this as a physiological response and have outlined what is presently described as "nucleotide permeabilization." ATP was shown to cause large, abnormal channels through which many substances (proteins, ATP, etc.) could pass. This phenomenon, however, appears to be peculiar to transformed cell lines and to occur only at high ATP concentrations ($>100 \mu\text{M}$) and at alkaline pH. An exception is the dog red blood cell (328), which at normal pH underwent permeability changes, but at high ATP concentrations ($>100 \mu\text{M}$).

Nucleotide efflux via a transporter protein is rare. One example is the classical system for elevating cAMP in the extracellular milieu in Dictyostelium discoideum (164,327). In this system, cAMP functions as a chemotactic agent for cell-to-cell communication. These cells also release ATP, the appearance of which is sensitive to potassium cyanide and dinitrophenol (327). The authors speculated that this ATP release may have occurred by a process similar to cAMP transport.

Release of cyclic AMP also occurs in nucleated avian red cells. The initial results concerning this nucleotide release demonstrated that epinephrine and isoproterenol stimulated, while probenecid inhibited, release (68). Subsequent research demonstrated that this nucleotide translocator was distinct from transporters of nucleosides, sugars, amino acids, cations and anions (142). Others (26) have shown inhibition of cAMP release

by prostaglandin A₁ (PGA₁). PGA₁ had no effect on adenosine transport. An inhibitor of the adenosine transporter, nitrobenzylthioinosine (NBFI), likewise had no effect on cAMP transport (201). The authors speculate that the cAMP translocator may have broad specificity. If this is true, then it may be just a matter of time before carrier-mediated ATP efflux is demonstrated.

The release of ATP from tissues may, therefore, involve four processes:

- 1) neurotransmitter release (exocytosis).
- 2) flow down an electrochemical gradient, possibly through a channel in response to hypoxia or as a result of membrane depolarization.
- 3) carrier-mediated translocation across the membrane.
- 4) "nucleotide permeabilization."

When ATP appears in the extracellular space, hydrolysis is extremely likely because of the high concentration of ecto-enzymes associated with various tissues.

Control of Extracellular Nucleotide Concentration: Ecto-enzymes

In general, ecto-enzymes must fulfill specified criteria (20,283,334,410). They must be an integral protein of the plasma membrane and their active site must be externally directed to act on a non-penetrating substrate and be antagonized by non-penetrating inhibitors. These criteria are satisfied by ecto-enzymes that are abundantly situated to deal with extracellular levels of ATP (20). For instance, cardiac muscle has considerable ability to degrade ATP and adenosine that is

perfused through it (154,229,421). One enzyme, 5'-nucleotidase, is especially abundant on almost all myocardial extracellular surfaces, and ATPase and ADPase activity is high in the heart (335,387). Skeletal muscle has been shown to sequentially degrade exogenous ATP to IMP, using an ATPase, adenylate kinase and AMP deaminase (128,283,285,473). A 5'-nucleotidase is also present, which results in extracellular adenosine production.

Vascular smooth muscle and endothelial cells have also been shown to hydrolyze exogenous ATP to adenosine, using ecto-enzymes with different specificities (91,115,120,165,184,199,336,338,375). Though no deaminase or ATP-pyrophosphatase activity exists, both cell types have the following ecto-enzymes:

- 1) magnesium-stimulated nucleoside triphosphatase (non-specific: $ATP \rightarrow ADP + P_i$, or $XTP \rightarrow XDP + P_i$)
- 2) nucleoside diphosphatase ($ADP \rightarrow AMP + P_i$)
- 3) nucleoside diphosphate kinase ($2 ADP \rightarrow ATP + AMP$)
- 4) 5'-nucleotidase ($AMP \rightarrow adenosine + P_i$).

Adenosine deaminase activity (adenosine \rightarrow inosine) has also recently been demonstrated (202,303).

Due to the vast surface area of red blood cells, the presence of ecto-ATPases in their membranes constitutes a significant provision for hydrolysis of ATP (438,442). Individual human red cells are said to degrade 3.2×10^6 molecules of ATP per minute (206), a figure that is estimated to be about 1000 times higher than that for nucleated avian red cells (443). Ecto-ATPase activity of frog erythrocytes is 37 nmoles/ml cells \cdot min (438), or 1.86×10^7 molecules of ATP per cell per minute (see p. 40).

Ecto-enzymes in muscle, red cells, and vascular tissue makes it likely that they function to control the local concentration of ATP even to the point of degrading some of it to a substance (adenosine) that is vasodilatory, but a thousand times less potent than ATP. The production of adenosine by ecto-enzymes, particularly 5'-nucleotidase, is now considered to be integral to the adenosine hypothesis (311,373). This enzyme, of course, must have a source of AMP.

Schrader (386) has shown that perfusion with α,β -methylene ADP to inhibit 5'-nucleotidase activity had no effect on the hypoxia-induced release of adenosine from the guinea-pig heart. This seems to indicate that the adenosine appearing in the coronary circulation did not originate extracellularly. Yet, in this type of experiment it is always questionable whether the perfusate reached the interstitial space to inhibit, in this case, all of the 5'-nucleotidase enzymes.

Other cells also exhibit ecto-enzymes for ATP degradation: mast cells (73,92), white blood cells (113,118,139,293,349,409, 431,472,401), platelets (74,288,449), thymocytes (119), mammary cells (70), kidney tubules (365) and rat adipocytes (318,409). The central nervous system also has considerable ability to degrade ATP.

In the rat hippocampus, 5'-nucleotidase was not located at all synapses, but when present it was integral to both the

pre- and post-synaptic membranes (31). An important consideration concerning these ecto-enzymes is that their activity varies as a function of cell differentiation and growth (410): activity is lowest during mitotic activity and in immature cells.

ATP Content and ATPase Activity of Erythrocytes

The nucleotide concentration of the interstitial space is an unknown quantity. The plasma, on the other hand, is generally thought to have a concentration of ATP of 1 μ M or less (9,150,192, 329,387,464) except during exercise, when it is usually higher (144,150,329). Trauma (177,438) and localized or disseminated platelet activation (44,90,224,263) can also change nucleotide levels radically. For example, not only is ATP released from damaged tissue, but when plasma concentrations exceed 10^{-4} M, blood elements release intracellular ATP by a process similar to the aforementioned "nucleotide permeabilization" (438). Some membrane "shedding" during normal circulation (127) may partially account for basal levels of adenine nucleotides in the plasma. Loss of ATP from red blood cells is not considered to be a normal occurrence. Rather than lose ATP, the red cell mass functions as a sink for adenosine (330,331,345,389) in the same genre as the endothelium (336,315).

Mature human red blood cells, anucleate and without mitochondria, rely on glycolysis as their predominant source of ATP production (389). With an oxygen consumption of

0.3 μ moles O_2 /ml cells·hr, human red cells produce and maintain millimolar levels of ATP (35,36,37,38,39,45,48,49,50,51,52,94, 108,130,182,302,341,354,385,389,417,435,461). Genetic deviations or disease processes such as anemias, lymphosarcoma, and pulmonary abnormalities result in concentrations of ATP that are decreased or sometimes elevated (48,50,51,130,302). Cell ageing (50,51,284,309,310) also causes ATP depletion in red cells. Table I documents the ATP concentration of human and frog red cells, the method by which it was determined, and the reference.

The oxygen-carrying capacity of frog red cells is 10.3 vol.%, about half that found in human red cells (276). In addition to the decreased oxygen-carrying capacity, nucleated cells consume considerably more oxygen than do cells without a nucleus.

The nucleated cells of amphibians, fish and birds consume O_2 at a rate greater than 2 μ moles O_2 /ml cells·hr (389), which is about six times greater than that of human red cells. This is due to the higher concentration of glycolytic and tricarboxylic acid cycle enzymes and especially to the presence of active mitochondria which produce ATP via oxidative phosphorylation (389,103) in nucleated red cells. The spleen is the primary erythropoietic organ in the adult frog (15), producing flattened, elliptical, disc-shaped cells. These erythrocytes undergo mitosis in circulation (15,384), and maintain millimolar ATP levels that are allegedly higher than that observed in the human red cell (24,389).

TABLE I
Erythrocyte ATP Concentration (in mM)*

<u>Adult Human</u>	<u>Assay Method</u>
2.84 (461)	chromatography
4.76 (37)	firefly
3.04 (39)	chromatography
3.69 (45)	HPLC
3.36 (94)	HPLC
2.14 (108)	HPLC
5.76 (36)	firefly
1.10 (435)	NMR
4.08 (182)	hexokinase
4.28 (38) caucasian	firefly
3.86 (38) black	firefly
4.47 (38) caucasian	hexokinase
3.73 (38) black	hexokinase
4.02 (130)	?
3.89 (50)	hexokinase
5.49 (385)	HPLC
4.39 (302)	fluorescence assay
3.28 (48)	?
2.91 (51)	hexokinase
3.67 (49) caucasian	hexokinase
3.35 (49) black	hexokinase
4.33 (417)	HPLC
3.87 (354)	?
5.68 (52)	HPLC

<u>FROG</u>	<u>Assay Method</u>
6.10 (24)	chromatography
3.31 (52)	HPLC

*See APPENDIX I for calculations. Number in parentheses is the appropriate reference. HPLC: high performance liquid chromatography. Hexokinase refers to the coupled assay hexokinase-glucose-6-phosphate dehydrogenase, with NADPH measured.

Besides functioning as an energy source, red cell nucleotides interact with hemoglobin to alter the affinity of hemoglobin for oxygen. This is classically described in human red cells, which produce 2,3-diphosphoglycerate (2,3-DPG) as the allosteric effector of oxygen-hemoglobin binding (26,27,76). Assuming only moderate hypoxia of the hypoxic type, 2,3-DPG functions as a cofactor by binding to hemoglobin to shift the oxyhemoglobin curve to the right (277). In this way, 2,3-DPG increases the efficiency of oxygen delivery by causing hemoglobin to have less affinity for oxygen and encouraging O_2 off-loading to the peripheral tissues. For an aerobic organism like the human, whose tissues are very oxygen dependent but whose respiratory capacity is high, this capability for rightward shift in the oxyhemoglobin dissociation curve provides considerable aerobic reserve and allows humans access to virtually all of the earth's land mass at practically all altitudes.

Organic phosphates affect the affinity of hemoglobin for oxygen the same way in most animals. The organic phosphate cofactors, however, vary in their importance based on their concentration, and the concentration varies by specie. Any one or a combination of 2,3-DPG, ATP, guanosine triphosphate (GTP) or inositol pentaphosphates (IP₅) (23,200,451,452) may function to decrease hemoglobin affinity for oxygen. The developmental stage of red cells is important to the type and concentration of phosphate compound present (23). ATP is most important in the adult frog.

The decrease in hemoglobin-oxygen affinity caused by these substances can be rank ordered: $IP_5 > GTP > 2,3\text{-DPG} > ATP$. The concentration of these substances in the red cells of animals is relatively high in accordance with the degree of allosterism observed. One may expect to find significant shifts of affinity for oxygen in birds, which have high IP_5 , but other animals will have smaller affinity shifts according to the organic phosphate that predominates in their red cells: mammals with 2,3-DPG, fish with GTP and ATP, and amphibians with ATP.

In fish (351,451,471) and eels (470), and presumably in frogs, environmental changes in the oxygen partial pressure (PO_2) and/or temperature are commonly encountered, and result in changes of organic phosphate level. Rather than an increase, as found in humans, the organic phosphate level decreases with decreased pO_2 . Frogs submerged in hypoxic, stagnant water (e.g., during hibernation in the mud), adapt to low oxygen by increasing the hemoglobin-oxygen affinity. This is caused by decreased ATP. As blood moves through the organs of gas exchange (e.g., in frogs, the rudimentary lungs, the buccal-pharyngeal membranes, and the skin) the animal is able to survive aerobically because the oxyhemoglobin dissociation curve is shifted to the left, enhancing the on-loading of oxygen (366) from the hypoxic environment.

The phenomenon of loss of organic phosphate in response to decreased environmental oxygen entails one of the intermediate responses of the organism to hypoxia. Additional responses to the hypoxic insult include splenic release of erythrocytes, increase in hematocrit and decrease in plasma pH (351). If unable or unwilling to move to a more normoxic water environment, and having acutely responded with changes in heart rate and respiration, the organism's blood is able to provide a defense against hypoxia within hours by reducing the erythrocyte organic phosphate concentration. Metabolic readjustments within the erythrocyte undoubtedly occur (351,174), but are probably not the sole means for reducing red cell organophosphates (see DISCUSSION). Evidence exists that hypoxia-induced reduction of ATP in red cells of a fish, Fundulus heteroclitus (Bull minnow), occurs acutely within the first hour (175). This same observation in Squalous acanthius (dogfish) has been correlated with a reduction in the P_{50} during the first hour following hypoxic insult (455).

Red blood cells also demonstrate the ability to hydrolyze extracellular ATP (see p. 33). If ATP appears in the plasma as a consequence of injury, cell lysis due to age, neurotransmitter release, hormonal secretion, or in the course of normal cell function, its concentration must be controlled due to its vasodilatory effects and the fact that it can cause permeabilization. This latter phenomenon appears to be less critical for nucleated red blood cells of animals like the

frog and eel. Resistance to permeabilization may be due to the lipid composition of these erythrocytes, which have a higher phospholipid content than mammalian red cells (168).

The ATPase activity of nucleated red cells varies depending on the species (438). Table II provides a comparison of the rates at which red cells of various animals can hydrolyze extracellular ATP. This ecto-ATPase activity may be crucial to limiting the effects of ATP in traumatic shock (438) or in decreased peripheral resistance. These ecto-enzymes also may function as part of the normal adaptation by organ systems to environmental changes and in cell-to-cell communication.

Ethylenediaminetetraacetate (EDTA) has reportedly been a strong, reversible inhibitor of the ecto-ATPase activity of nucleated red blood cells (443). Since these hydrolyzing ecto-enzymes are directed outward from the plasma membrane, non-penetrating reagents can selectively inhibit them. Such reagents include suramin, sulphanilic acid, 5'-nucleotidase antibodies, α, β -methylene ADP and concanavalin A (334). Though specificity may preclude direct inhibition of the ecto-ATPase (e.g., using 5'-nucleotidase antibodies), alteration of the local environment may cause a modification of enzyme activity.

TABLE II
Ecto-ATPase Activity of Various Erythrocytes*

Animal		ATP Molecules Hydrolyzed cell ⁻¹ min ⁻¹
eel	(438)	3.31 X 10 ⁷
frog	(438)	1.86 X 10 ⁷
man	(206)	3.20 X 10 ⁶
man	(438)	3.82 X 10 ⁵
rat	(438)	5.61 X 10 ⁵
rabbit	(438)	4.39 X 10 ⁵
cat	(438)	1.54 X 10 ⁵
pigeon	(443)	2.00 X 10 ³

*See APPENDIX II for calculations. Number in parentheses is appropriate reference.

However, altering the local environment has not always proved to be an effective technique. For example, neuraminidase treatment does not change the activity of ecto-ATPase and 5'-nucleotidase in certain cells (439). Conversely, neuraminidase treatment resulted in the release of ATP and ribonucleic acids (439).

Inhibition of ATPase may occur only from direct competitive interaction of a reagent with that portion of the enzyme protein projecting from the cell surface, or in the case of EDTA, by removing a required cofactor, such as magnesium (443). No specific inhibitor of ecto-ATPase has been found, but trifluoperazine (293), ethacrynic acid (73), N-ethylmaleimide (401), and difluorodinitrobenzene (377) have been used with some success in inhibiting ecto-ATPases of some cells. Whether these substances inhibit the ecto-ATPase on red blood cells has not been determined.

The prevailing evidence is that the outside surfaces of cells are able to hydrolyze ATP. These include cells from skeletal muscle, vascular smooth muscle, endothelium, red and white blood cells, platelets, and synaptic membranes of neurons.

CALCIUM MOBILIZATION IN CARDIAC CELLS: VOLTAGE-SENSITIVE
VS. RECEPTOR-MEDIATED MECHANISMS

ATP is able to mobilize calcium in the myocardium (see p. 54). Therefore, a discussion of calcium homeostasis and mobilization into the cell is appropriate.

Control over calcium inside the cell is essential for cell life. Besides conveying partial excitability to cells like muscle and nerve, calcium provides stability and regulatory control to various membrane proteins, e.g., integral proteins of both the cell membrane and membranes of internal organelles. With calcium maintained at a low intracellular concentration, it exhibits a permissive role in allowing phosphate-driven metabolism.

Calcium also can function as a second messenger. Due to its low concentration, the influx of a few calcium ions can be used to activate enzymes. Recovery of these few ions to the cell exterior or into an organelle is accomplished at the expenditure of a relatively small amount of energy. None of the aforementioned would be possible were it not for the various mechanisms for entry and exit of small amounts of calcium.

The following discussion is devoted to contrasting the entry of calcium via voltage-sensitive channels and mobilization of calcium via receptor-mediated mechanisms. Control over cell calcium is briefly reviewed. Particular emphasis is placed on the recent electrophysiological evidence for calcium channels in myocardial cells, and on the recent hypothesis that activation of phospholipids from the cell membrane causes elevated calcium in

the cytoplasm. Calcium blockers are introduced to demonstrate how they may be used to unravel channel control mechanisms.

Control of Intracellular Calcium

Cells exhibit precise control over their internal concentration of calcium. For this reason, calcium influx can and does function to initiate various cellular functions when its concentration exceeds basal levels of approximately 100 nM. This "resting" concentration is maintained by various mechanisms (69, 167, 287):

- 1) a calcium pump in the plasmalemma that is ATP dependent.
- 2) a sodium-calcium exchanger in the plasmalemma that is dependent on the sodium gradient.
- 3) a calcium pump in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER) membranes that is ATP dependent.
- 4) a calcium uniporter in the inner mitochondrial membrane that is dependent on the negative membrane potential generated by proton transport.

As a result of low intracellular calcium, very few influxing calcium ions are necessary to activate or inhibit enzymes, and very little energy has to be expended to re-establish resting levels.

Low calcium is crucial for at least two other reasons. First, high-energy phosphate-driven metabolism would be impossible without low intracellular calcium because inorganic phosphate from ATP hydrolysis would precipitate into hydroxyapatite crystals. Second, gating of some membrane channels would be impossible. Channels that are regulated, i.e., opened or closed, in response to changes in the transmembrane potential are very dependent on

low intracellular calcium levels and minor fluxes into and out of the cell.

The mechanisms by which calcium enters the cytoplasm are varied. Non-specific diffusion down an electrochemical gradient is possible. Voltage-sensitive calcium channels exist in the plasmalemma. Other calcium channels in the plasmalemma, SR and ER may account for considerable flux of calcium. Mitochondria have a sodium-calcium antiport which exchanges one calcium ion for two sodium ions. Thus, a number of mechanisms exist for allowing an influx of calcium into the cell.

A rapid and large increase (10 to 100-fold) in cytosolic calcium can provide almost instantaneous communication, as seen on a beat-to-beat basis in cardiac muscle. In adult hearts, calcium influx provides the signal for the release of some contractile calcium from the SR (136). The frog heart is an exception. Calcium-induced release of calcium from the SR is not necessary for excitation-contraction coupling (135,136). The frog heart is apparently the only exception among the various adult cardiac tissues studied. Frog ventricle derives its contractile calcium from the extracellular pool upon depolarization of the plasmalemma (135,136,258,319). The consequence of this is that the calcium of the SR is not recycled, the calcium concentration of the SR cannot be increased, and no post-extrasystolic potentiation can be elicited (258).

Calcium Channels in Myocardial Cells

A few years ago, only one calcium channel was discussed. Now the debate involves how many different calcium channels there are, the manner by which they are distinguished in function and distribution (neuron vs. muscle, for example), which type is sensitive to the various calcium channel blockers, and which are responsible for the physiological currents measured (207,251,292,300,323,358).

Voltage-sensitive channels of the cell membrane are the ones best described. Under voltage clamp conditions and with the use of sodium and potassium blockers, it has been possible to isolate calcium currents. By comparison with the channels that convey sodium ions with currents of $1-5 \text{ mA/cm}^2$, calcium channels, even when maximally activated with excess calcium or barium present, elicit currents less than $100 \mu\text{A/cm}^2$ (209). Their rate of activation is slow, but rapid inactivation occurs upon repolarization (209).

The calcium channel that appears to be most common has been designated the L-type (207,323,358). This channel was found in both muscle and nerve cells. It decayed slowly ("L" for long lasting with a $t_{1/2}$ of hundreds of milliseconds), required strong depolarizations (voltage steps $> 40 \text{ mv}$), and was sensitive to dihydropyridine antagonists and the agonist Bay K 8644. Average current size (from isolated single channels in patch clamp) was approximately 2 pA.

The other two calcium channels have been designated T (for transient) and N (for neither T nor L). These channels were not sensitive to dihydropyridines. Average unitary currents for T and N were 0.62 pA and 1.22 pA, respectively, and they demonstrated time-dependent inactivation. By contrast, L channels were relatively resistant to inactivation.

Though this information primarily comes from the study of dorsal root ganglia in chick embryo (323), unitary T and L channels have also been studied in myocytes from the ventricles of guinea pigs (207). In addition to verifying that the L channel was affected by nimodipine and Bay K 8644, this research also demonstrated that barium was conducted through the L channel more efficiently, resulting in a larger current. Additionally, current through T channels was preferentially retained following excision of a membrane patch during patch recording, whereas the current through L channels is quickly lost. Some intracellular constituent is apparently essential for normal function of L channels.

Recent research also identified the sodium-calcium exchange current in single cardiac myocytes (251,292). It was electrogenic and voltage-dependent, required intracellular free calcium, resulted from release of calcium from the SR, and was blocked by lanthanum. It has been suggested that this current may contribute to pacemaker activity (292).

Receptor-Mediated Calcium Mobilization

Receptors that either stimulate or inhibit the adenylyl cyclase-cAMP system have been well characterized. However, the total cellular response (i.e., which enzymes and substrates are affected) has not been totally elucidated. In the heart epinephrine (via β receptors) has positive inotropic and chronotropic actions (398). This occurs when the cAMP-pathway modulates intracellular calcium. The same agonists that affect

the cAMP pathway in one tissue may produce different actions in another tissue due to a different receptor and pathway. For example, a series of changes in cell function can occur concurrently with or following the hydrolysis of phosphatidyl-inositol (PI) (32,33,34,67,167).

Due to the nature of the adenylyl cyclase-cAMP pathway, several criteria must be defined for classifying the receptors involved in calcium mobilization via phospholipid pathways (167).

They are as follows:

- 1) Calcium influx or efflux must correlate with agonist-receptor binding.
- 2) Calcium ionophores must effect responses similar to agonist-mediated responses.
- 3) Absence of calcium must prevent the response.
- 4) Adenylyl cyclase must not be affected by the agonist-receptor binding.
- 5) Guanine nucleotides must not alter receptor binding.
- 6) The receptor-mediated response must occur independent of any changes in cAMP levels.
- 7) Phosphatidylinositide metabolism must correlate with agonist-receptor binding.

When these criteria are met, what other events take place upon stimulation of a calcium-coupled channel?

As the result of an agonist-receptor interaction, various phospholipids in the cell membrane are enzymatically altered. For instance, phosphatidylethanolamine (PE), which predominates in the inner leaflet of the cell membrane, becomes methylated (with S-adenosylmethionine) to phosphatidylcholine (PC), with subsequent translocation to the outer leaflet (32,67). It has been suggested (67) that four events may then result:

- a) a change in membrane fluidity, with enhanced interaction of integral proteins (i.e., a G protein with adenylyl cyclase).
- b) up or down regulation of cell receptors.
- c) increased calcium permeability.
- d) activation of phospholipase A₂.
- e) release of arachidonic acid (AA) from phospholipids.
- f) prostaglandin synthesis as a result of AA release.

Calcium-coupled receptors also are responsible for other cellular effects, in particular, the turnover of phosphoinositide lipids (32,67,123,167,210,214,297,321). This observation dominates the other criteria of calcium-coupled receptors, causing certain structural and functional changes in the cell. First, PI may be hydrolyzed to diacylglycerol (DG) and/or inositol 1,2-cyclic phosphate and inositol 1-phosphate. The enzyme responsible for this hydrolysis is a PI-phosphodiesterase, also called phospholipase C.

Besides PI, the inner leaflet of the cell membrane contains proportionally less concentrations of phosphatidylinositol 4-phosphate (diphosphoinositide, or DPI) and phosphatidylinositol 4,5-diphosphate (triphosphoinositide, or TPI or PIP_2). Given a particular cell type, the breakdown of PI, DPI, and TPI may or may not be dependent on calcium (32). However, it is TPI which appears to be preferentially degraded, releasing DG and inositol 1,4,5-triphosphate (IP_3) (33,34,167). With TPI representing about 6% of the hormone-sensitive pool of phosphoinositides, agonist-mediated turnover of each of the phosphoinositides exhibits its most significant effect by producing IP_3 from TPI (34).

As reviewed by Berridge (34), IP_3 may function as a second messenger to cause the release of calcium from internal stores. He cites as evidence the release of calcium from a non-mitochondrial pool, possibly the ER, upon the application of IP_3 to permeabilized liver, smooth muscle, and pancreatic cells. Recent evidence in adrenal chromaffin cells also supports this hypothesis (418).

Although the IP_3 -second messenger pathway is strongly supported by the evidence to be the pre-eminent cause of calcium release, additional events may result from phosphoinositide turnover and be just as important (32,34,67,167).

- 1) DG may be phosphorylated to phosphatidic acid (PA). PA may then act as a calcium ionophore to allow calcium influx, either through the cell membrane or ER membrane. There is also some evidence that this branch of the pathway may provide some auto-regulation via calcium pumps, decreased IP₃ production, and receptor modulation (125).
- 2) Calcium bound to PI in the cell membrane may be released with the hydrolysis of PI, which may open a voltage-sensitive calcium channel.
- 3) Hydrolysis of the phosphoinositides may release AA, an abundant acyl group in the 2-position of phospholipids. Various eicosanoids may then be formed from AA to result in varied cell-specific actions, including calcium release.
- 4) Guanylate cyclase may be activated. The mechanism is not known, but may involve AA or a metabolite of AA.

The association of these various events with one another and with agonist-receptor activation still only provides circumstantial evidence for a cause-effect relationship. It is possible that these events also may occur concurrently.

Calcium-Antagonist Effects

Establishing which substances antagonize which calcium actions will better define the roles that calcium plays in normal cell functions. It appears that at low doses calcium antagonists preferentially affect voltage-sensitive channels (167,313). The fact that they can affect agonist-mediated mechanisms of calcium mobilization does not exclude an association between both mechanisms of calcium release. In some instances the mechanisms for voltage- and receptor-mediated calcium mobilization may involve the same calcium channel, though substantiation of this hypothesis has not yet occurred.

As already discussed with regard to voltage-sensitive channels, it appears that the dihydropyridines are effective inhibitors of L channels, but not N and T channels. Evidence exists that calcium antagonist binding sites are functionally associated with the channel (131). Thus, different molecular structures for the three calcium channels can be surmised (131). This is supported by the fact that the binding site of dihydropyridines is distinct from the binding sites of verapamil and diltiazem (300).

A model exists (388) of a voltage-sensitive calcium channel that takes into consideration the effects of the calcium antagonists. At the channel entrance are negative charges indicative of the binding sites for the inorganic cations which can block calcium entry. These inorganic blockers include manganese, lanthanum, magnesium, nickel and cobalt, all of which antagonize calcium entry by displacing it from these obligatory binding sites (13,198,274,312). Closer to the voltage-sensitive gate, but external to it, is the binding site for the organic calcium-antagonist, nifedipine. Characteristic of the dihydropyridines, nifedipine acts as if it were a "plug" in the channel. It is therefore effective whether or not depolarization continues to occur (47,304,388). Alternatively, verapamil and diltiazem attach to binding sites internal to the gate. Their ability to antagonize calcium entry is "use-dependent", i.e., depolarization must occur to allow access to their binding

sites (47,274,312,388). Consequently, interference with calcium entry by these blockers only occurs once the gate is open.

These observations combined with evidence of calcium-channel activators has led to the proposal of three modes of gating activity (388). Thus, conditions characteristic of calcium currents of short duration is described as Mode 1. Mode 0 exhibits no calcium currents and Mode 2 displays currents which are long-lasting. Mode 0 can be achieved with blockers and Mode 2 with activators such as Bay K 8644, CGP 28392, and YC 170 (388). These calcium channel activators are chemically similar to the dihydropyridines, bind at the "N" binding site, but function in such a way as to increase the probability of channel opening.

Calcium Mobilized by ATP

Due to an excitatory receptor, the P₂X purinoceptor (171), ATP produces a positive inotropic effect which is striking in the isolated frog heart (445). This response to extracellular ATP has been described as triphasic: an initial, abrupt increase in contractility, a secondary decrease, and finally a sustained increase above control values (140). The effect of ATP on the frog heart has been explained as follows. The duration of the action potential, as observed in isolated trabeculae, increases (140,320). In particular, phase 2 of the action potential (plateau phase) is prolonged (140,320). This is the first indication that there may be an association between voltage-dependent calcium channels and receptor-mediated release of

calcium. That is, ATP appears to affect calcium movement during the action potential, thereby causing increased availability of calcium via a voltage-dependent channel in the sarcolemma. This could occur as the result of the P_{2X} receptor increasing calcium conductance through a slow-inward channel, though activation of other latent calcium channels should not be ruled out.

Besides mobilization of extracellular calcium, ATP may also modulate intracellular levels of calcium via other mechanisms. Even without an imposed depolarization (evoked action potential), ATP is able to depolarize trabeculae by 10 mv and elevate the tension. The increased tension has been attributed to calcium release from the SR (320). Other evidence indicates that prostaglandins may participate in these actions. By comparison with a control response of isolated frog ventricle to ATP, when indomethacin at 10 μ M concentration is present the triphasic response is altered. The first phase of the response is diminished and the third phase of the triphasic response is eliminated altogether (140). It is obvious that a multitude of cellular effects occur when ATP activates its P_2 receptor.

Thus, two means for calcium mobilization can be contrasted in the myocardium: one via voltage-sensitive channels and one via receptor-mediated mechanisms. Existing evidence is too weak to conclude that the two processes are coupled. Calcium mobilization via voltage-sensitive channels is more sensitive, on the basis of concentration, to the calcium antagonists.

Presently, three voltage-sensitive channels are known, two of which (the L and T channels) also exist in the myocardium. With regard to calcium-coupled channels, the adenylyl cyclase-cAMP pathway is not the only second messenger system which influences intracellular calcium. IP₃ is another good candidate for mobilizing calcium from some internal store. Additionally, other events occur either concurrently with or subsequent to the metabolism of the phospholipids in the plasmalemma. Arachidonic acid, its metabolites, or cGMP may contribute to the receptor-mediated response.

A Potpourri of ATP Effects

The following are examples of the effects that extracellular ATP has on cells. Cell membranes change their permeability in response to extracellular ATP. This phenomenon is different from the "nucleotide permeabilization" process discussed earlier. Large channels are not formed and proteins are not lost to the cell exterior. In rat mast cells, ATP (1-40 μ M) induces a depolarization as sodium and potassium become permeable. This ultimately results in release of histamine (73,83,84,85,100). A similar permeability change ($K_m = 5 \mu$ M ATP) with potassium-specific efflux occurs in HeLa cells (7). In ascites tumor cells (414), a generalized permeability increase to ions was hypothesized as the cause of increased cell volume in response to extracellular ATP (0.6 mM). Also, Ehrlich ascites tumor cells increased their calcium uptake in response to ATP (2 mM) (273).

In isolated kidney tubules (365), ATP (1-4 mM) has been shown to cause calcium uptake and cell swelling. This cation permeability increase with ATP (0.1nM-1mM) has been associated with elevated inotropism in frog ventricle (140,398). It occurs early in cell development, having been observed in embryonic muscle cells (188) in response to similar concentrations (1-500 μ M). Sodium influx occurs in response to ATP (10 μ M-1 mM) in dog erythrocytes (364), which are high in sodium, but not in cat or human RBC's. This was hypothesized to occur as ATP interacted with membrane proteins. In isolated chromaffin granules (348) there was a documented change in the transmembrane potential induced by ATP (K_m = 40 μ M) causing a structural alteration in the membrane. This permeability change was theorized to be related to control of secretion. Calcium uptake also was stimulated in D. discoideum when ATP was present (327). Interestingly, these cells have the ability to provide their own extracellular ATP and cAMP. The purpose of the ATP is not known, but the cAMP is necessary for communicating with other D. discoideum during differentiation.

Extracellular ATP also has been shown to affect the metabolism of various cells. Millimolar concentrations stimulated glucose uptake and carbohydrate metabolism in kidney slices (453). More physiological levels (5-50 μ M) inhibited adipocytes from transporting glucose in response to insulin (75). Frog gastric mucosa could be prevented from secreting H^+ when 1-5 mM ATP was

present (378). The physiological significance of applying such a high concentration is dubious. However, the authors provide an interesting discussion concerning evidence for the possible uptake of ATP by these cells (129,247).

Extracellular ATP may communicate inhibitory information in certain cells. A cytostatic effect occurred at 40 μ M in certain tumor cells (355). This may be related to the low activity level of ecto-enzymes found in rapidly dividing cells (discussed earlier). Such a low activity may prevent these cells from hydrolyzing ATP, consequently precluding the cells from functioning normally. A similar observation was made in fibroblasts (233,259), which were prevented from aggregating in cell culture when millimolar ATP was present. Once ATP was hydrolyzed, cell aggregation returned.

Phagocytosis, a normal cell function of macrophages, could be prevented by the addition of millimolar levels of ATP (428). Another example is the stabilizing effect that could be produced by ATP (0.1-1 mM) on nerves (325), in addition to antagonizing the depressant effects of procaine (270).

Various stimulatory effects can be caused by ATP. In the cat, vasoactive intestinal polypeptide (VIP) release occurred in response to intra-arterial infusion of ATP in the gastrointestinal tract (399). Another example is less definitive. Though the mechanism is unknown, intraperitoneal injections of ATP (200 mg/Kg) increased the survival time of mice exposed to hypoxic environments (264,265,266). Perhaps this is related to glucose

mobilization that occurs in hepatocytes. Submicromolar concentrations of ATP in isolated hepatocytes are able to increase cellular levels of 1,4,5-inositol triphosphate with subsequent increases in intracellular calcium and phosphorylase activation (77,78).

Finally, synthesis of platelet activating factor (PAF) and prostacyclin (PGI₂) was shown to occur in endothelial cell cultures upon stimulation with ATP (1-300 μ M) (291,340). This co-production was demonstrated to be physiologically significant. The PGI₂ was produced briefly and released into the medium, whereas the PAF production was longer-lasting and remained in close association with the endothelium. This may serve in platelet aggregation to cause a localized activation via PAF release but still prevent disseminated intravascular coagulation via PGI₂ release.

The foregoing is a comprehensive review of ATP functions in the extracellular space. In addition to the well-known areas of neurotransmission and metabolic vasodilation, ATP appears to produce a variety of actions on many cell types. Collectively, these examples attest to the potent and pervasive means by which extracellular ATP can provide for cellular communication.

METHODS

ATP Assay

The method used for measuring ATP concentration in an aqueous solution was a modification of the firefly bioluminescence assay used by Strehler and McElroy (422). As shown in Figure 1, the procedure required the following equipment:

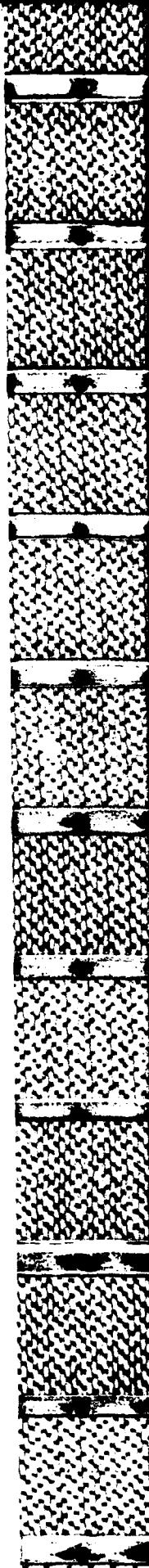
- 1) a photomultiplier tube (PMT) and sample holder,
- 2) a direct current power source for the PMT, i.e., a Hewlett-Packard 6515A DC power supply (1600 V),
- 3) a circuit breaker, and
- 4) a pen recorder, e.g., Fisher Recordall Model 5000.

Utilizing 100 μ l of reconstituted firefly lantern extract (FLE-50, Sigma) and 100 μ l of synthetic luciferin (D-luciferin, L-9504, Sigma), the reagents were micro-pipetted into four disposable cuvettes held by a carrier in the sample holder. The cuvettes were semi-micro size (Cole Parmer), having a transmittance of 400-800 nanometers and an optical path length of 10 mm. Each cuvette was cut approximately in half to fit the sample holder, holding a maximum volume of approximately 0.7 ml.

After loading the cuvettes into the carrier, the top of the sample holder was replaced. The energized PMT was only exposed to the transmission path of the cuvettes. The sample holder formed a seal that prevented room light from reaching the PMT but enabled assays to be conducted in a lighted room.

FIGURE 1
Equipment Used in the Firefly Bioluminescence Assay.

From left to right, the equipment includes: a Fisher Recordall Model 5000 pen recorder; the sample holder for the cuvettes (foreground) and container for the photomultiplier tube (PMT); the DC power source for the PMT, an H-P 6515A DC power supply (background); and a circuit breaker.



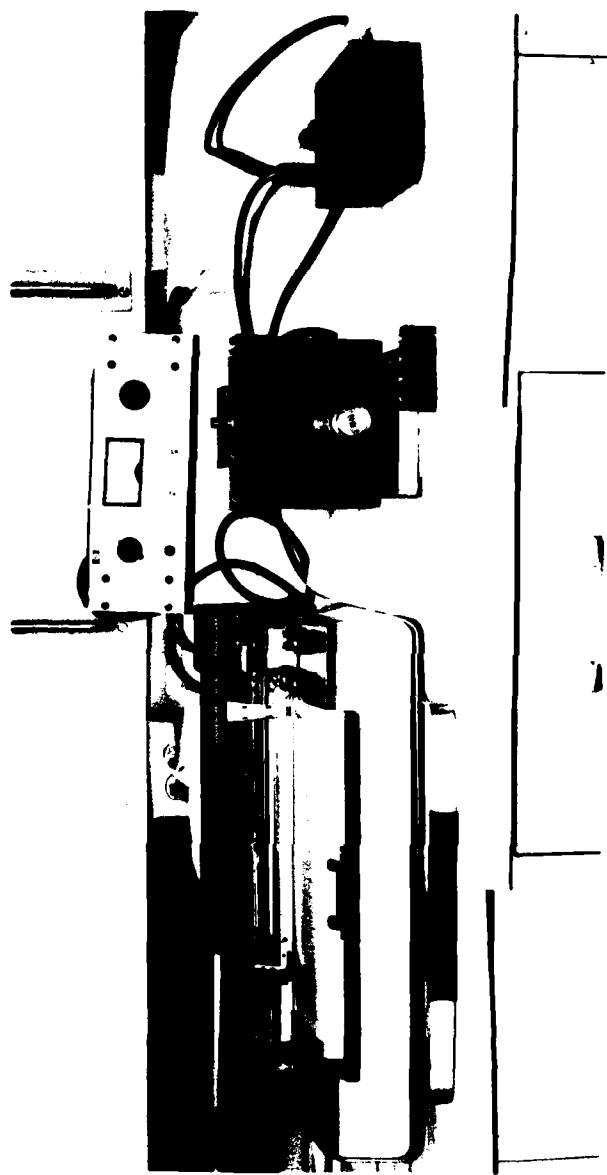


FIGURE 1

A sample volume of 200 μ l was injected into the injection port on the top of the sample holder, similar to the procedure used by Silinsky (394). Injection through the injection port required an SMI micropipette and a two-inch, 20 gauge hypodermic needle (Popper & Sons).

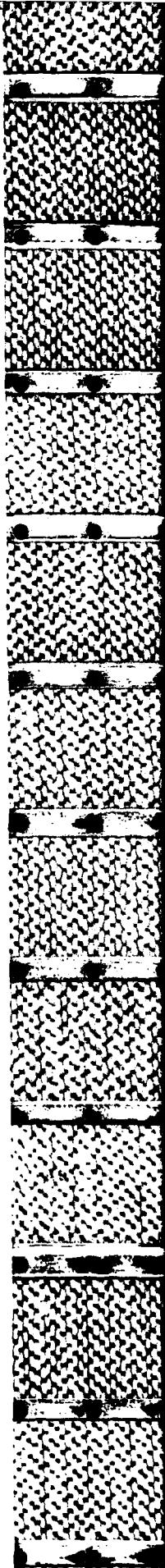
With 1600 V(DC) energizing the PMT, the presence of ATP in a sample could be detected by the amount of light emitted according to the enzymatic reaction (68,237) shown in Figure 2. The light signal was amplified by the PMT and recorded on the pen recorder in millivolts.

The recorded signal, shown in Figure 3, had two components. The initial signal, component A, was the background signal generated by the reaction mixture alone. Due to the presence of some endogenous ATP already in the firefly lantern extract (FLE), it proved beneficial to delay the assay procedure for 30 minutes until the background signal had become constant (see component A in Figure 3). Upon injection of 200 μ l of a sample or a known standard ATP solution, the reaction mixture was diluted, as demonstrated by the decrease in the recorded signal. This was followed by a rapid rise in the recorded signal as exogenous ATP was hydrolyzed (component B in Figure 3). This latter signal, the assay signal, was proportional to the concentration of ATP in the injected sample.

ATP concentrations of the unknown samples were obtained by comparison of the assay signals to a standard curve. This curve was created for each experiment as follows. Known concentrations

FIGURE 2
The Photinus pyralis (firefly) Enzymatic Reaction.

In the presence of excess luciferin (100 μ l synthetic luciferin), excess luciferase (100 μ l re-constituted firefly lantern extract), and with magnesium and oxygen present, light is emitted proportional to the concentration of ATP existing in a sample (200 μ l added to the cuvette containing the firefly reaction mixture). The light is emitted at a wavelength of 565 nm (yellow). It strikes the PMT, which enhances the signal, and in turn is recorded in millivolts on a pen recorder.



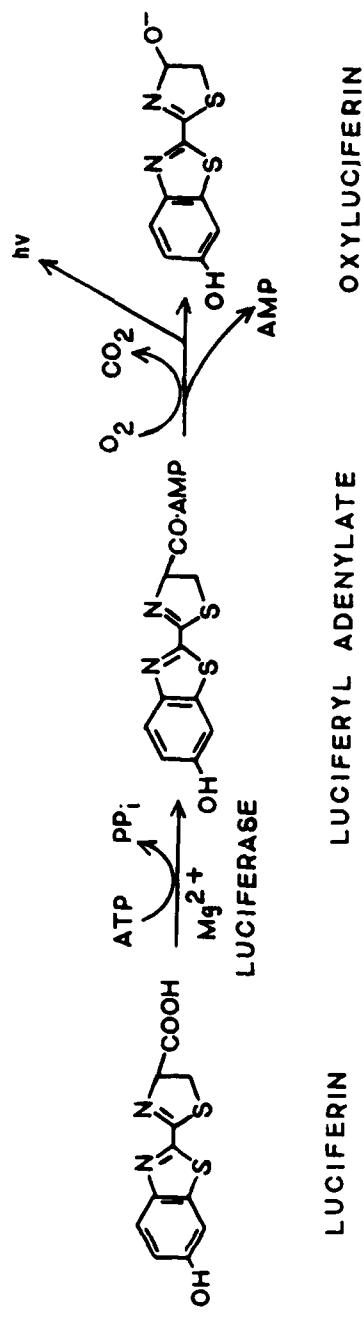


FIGURE 2

FIGURE 3
Emitted Light Signal Recorded from the Photomultiplier Tube.

Component A is the background signal emitted by the firefly reaction mixture. Upon addition of a sample to the mixture, component A is diluted. If ATP is present in the sample, component B (the assay signal) is generated. Component B exhibits a rapid-rise increase in the light signal and an exponential decay in addition to the peak that is proportional to the ATP concentration. Vertical bar, 10 mv; horizontal bar, 20 seconds.



FIGURE 3

of ATP (1 nM to 1 μ M) in frog Ringer's solution and a blank (ATP-free frog Ringer's solution) were assayed and the recorded signal plotted versus ATP concentration. The value of the recorded signal was calculated by dividing the assay signal (component B) by the background signal (component A) to give a signal-to-background ratio (B/A ratio). A blank sample (frog Ringer's solution without ATP) had a B/A ratio, and this was subtracted from each of the B/A ratios for all the known standards. A logarithmic plot of ATP concentration versus the B/A ratio resulted in a standard curve (Figure 4). After each unknown sample was assayed, its B/A ratio (minus the B/A ratio of the blank) was then plotted on the standard curve to determine the ATP concentration of the unknown sample.

By subtracting the B/A ratio of the blank, one has ensured that the standard curve is representative of the light signal emitted by the exogenous ATP and not as a consequence of the already existing ATP in the crude firefly lantern extract. Also, dividing the assay signal (component B) by the background signal (component A) ensured that the light signal emitted was comparable between all assays during an experiment.

FIGURE 4
ATP Standard Curve.

Known ATP standard solutions and a blank (ATP-free frog Ringer's solution) were assayed, generating light signals as in Figure 3. The ratio of component B to component A was calculated (B/A ratio), from which the blank signal (B/A ratio) was subtracted. This value was then plotted versus the ATP concentration. The curve is exponential and allows differentiation of two unknown samples in the nM range.

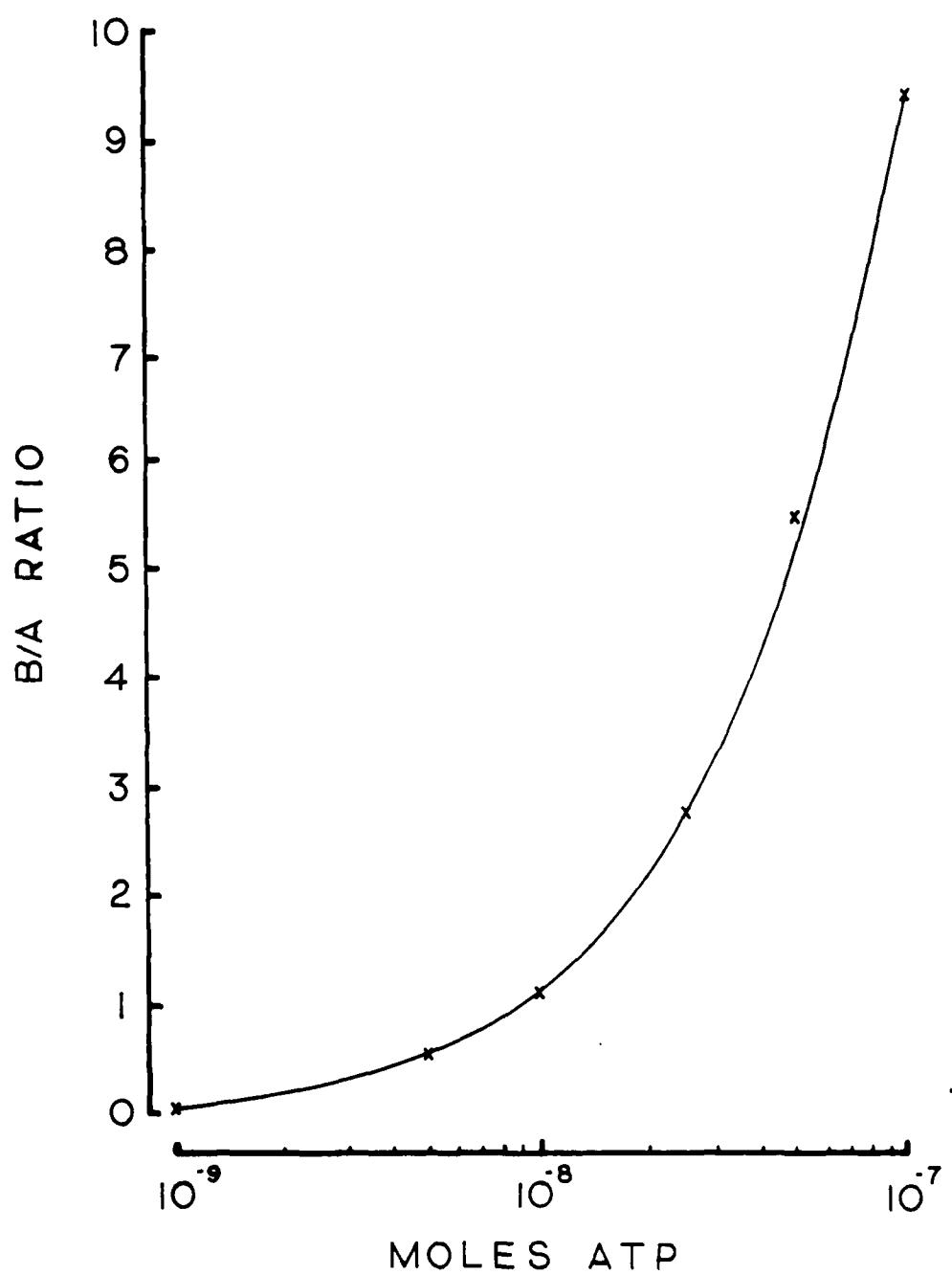


FIGURE 4

For example, the background signal (component A) of the firefly extract was observed to decrease over time, as shown in Figure 5a and 5b. This variability in the luciferase activity over time resulted in an assay signal (component B) that was diminished. Nevertheless, it exhibited the same proportionality to the background signal. Thus, if the B/A ratio was calculated at any given time, a comparison of ATP levels could be accurately made between samples. That is, time was not a factor in assaying the ATP concentration of the unknown samples. Figure 6 demonstrates that, using this procedure, there was no difference in the standard curves plotted with values acquired 80 minutes apart.

Effort also was taken to minimize signal loss due to quenching. Early experiments utilized quartz glass cuvettes, until it was shown that a mild degree of quenching of the light signal occurred. This is demonstrated in Figure 7. The inset shows that the recording of an ATP standard (5 nM) assayed in a glass (G) cuvette was very similar to a recording using a plastic (P) cuvette. Upon plotting the standard curves, however, the difference in the resultant light signal as measured by the B/A ratio became significant. The light emitted through glass

FIGURE 5
Light Signal Diminution With Time.

The activity of the firefly reaction mixture decreased with time, as seen when ATP standard solutions [1 nM in a) and 10 nM in b)] were assayed initially in "i", and again 80 minutes later in "ii". Both the background signal (component A) and the assay signal (component B) were diminished in amplitude. However, the B/A ratios were identical. Vertical bar, 10 mv; horizontal bar, 20 seconds.

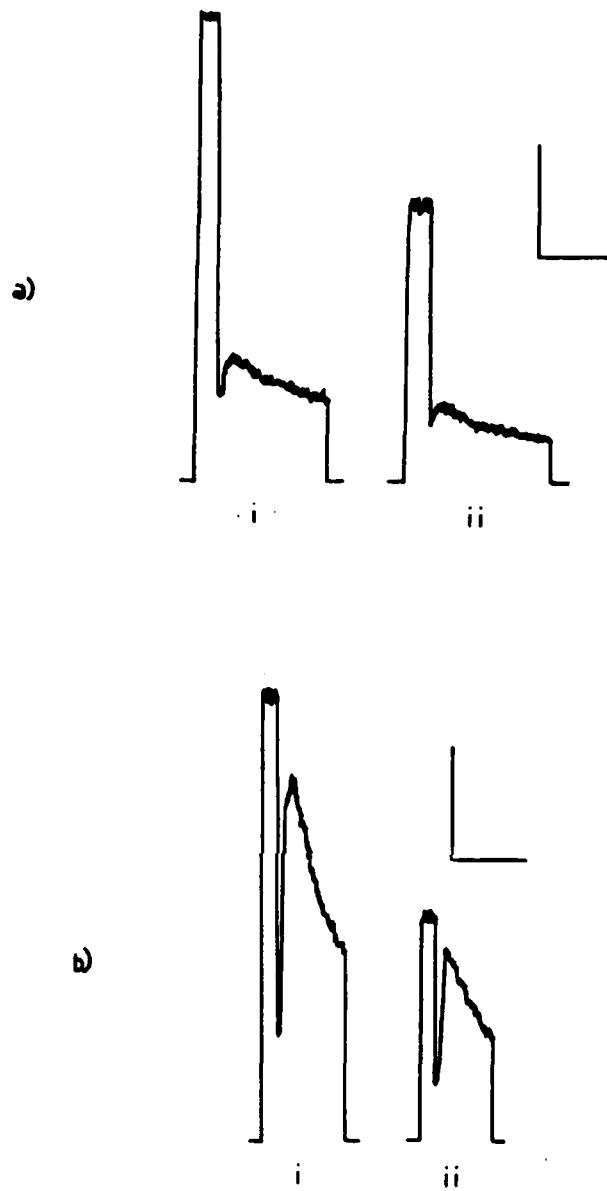


FIGURE 5

FIGURE 6

Comparison of ATP Standard Curves Generated 80 Minutes Apart.

B/A ratios calculated from signals as in Figure 5 are compared: x, first assay; o, second assay with the same ATP standard solution 80 minutes later. There was no difference in the standard curves. Therefore, time was not a factor in determining the ATP concentration of unknown samples assayed at different times.

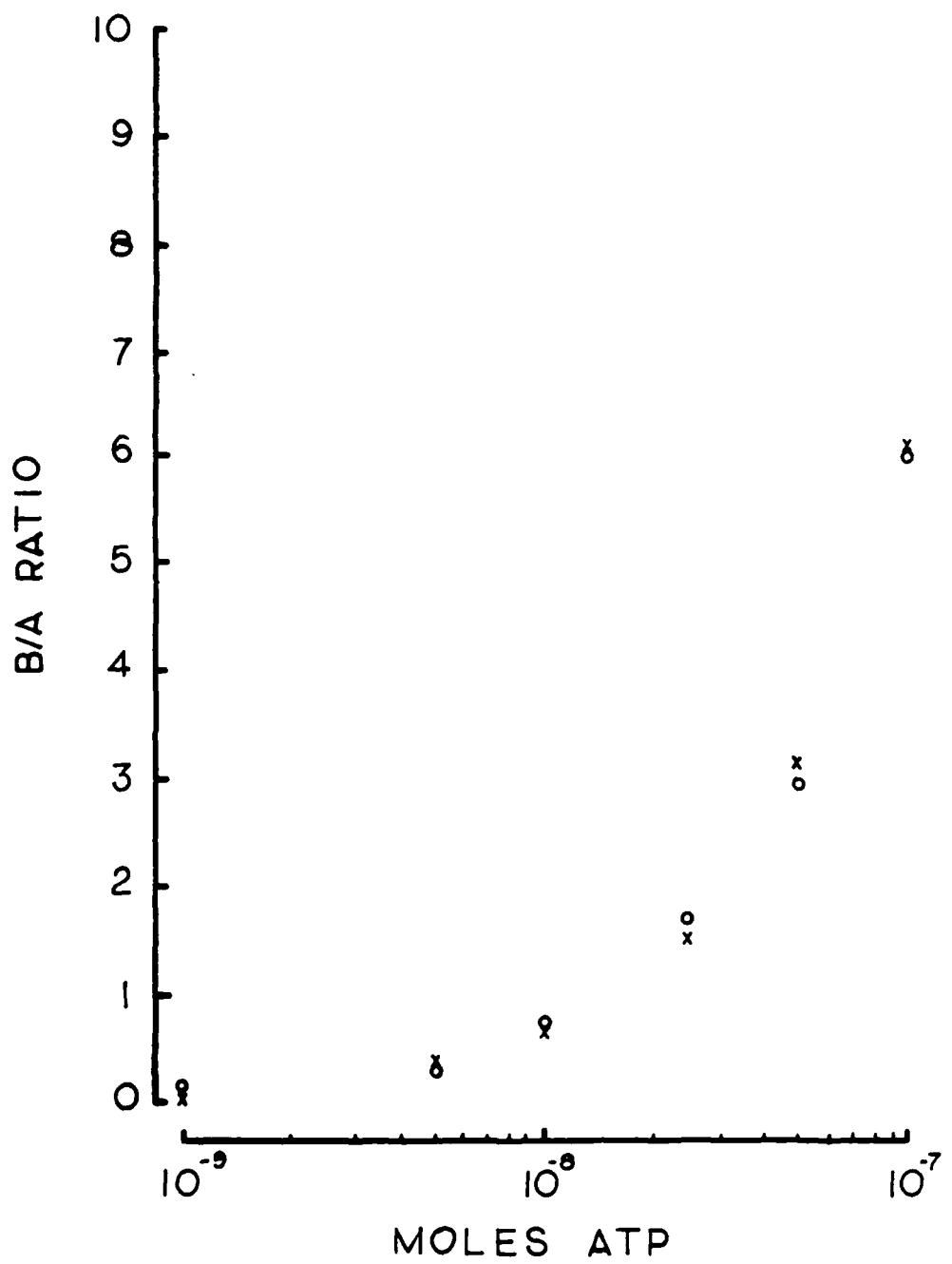


FIGURE 6

FIGURE 7

Comparison of Light Signals and Standard Curves When ATP Standard Solutions were Assayed Using Plastic (P) and Glass (G) Cuvettes.

The inset shows that light signals generated through plastic and glass appear to be the same. ATP concentration was 5 nM. Vertical bar, 10 mv; horizontal bar, 20 seconds. The standard curves were generated as in Figure 4. Assays through glass (o) did not emit as much light as through plastic (x), as seen by a comparison of the standard curves.

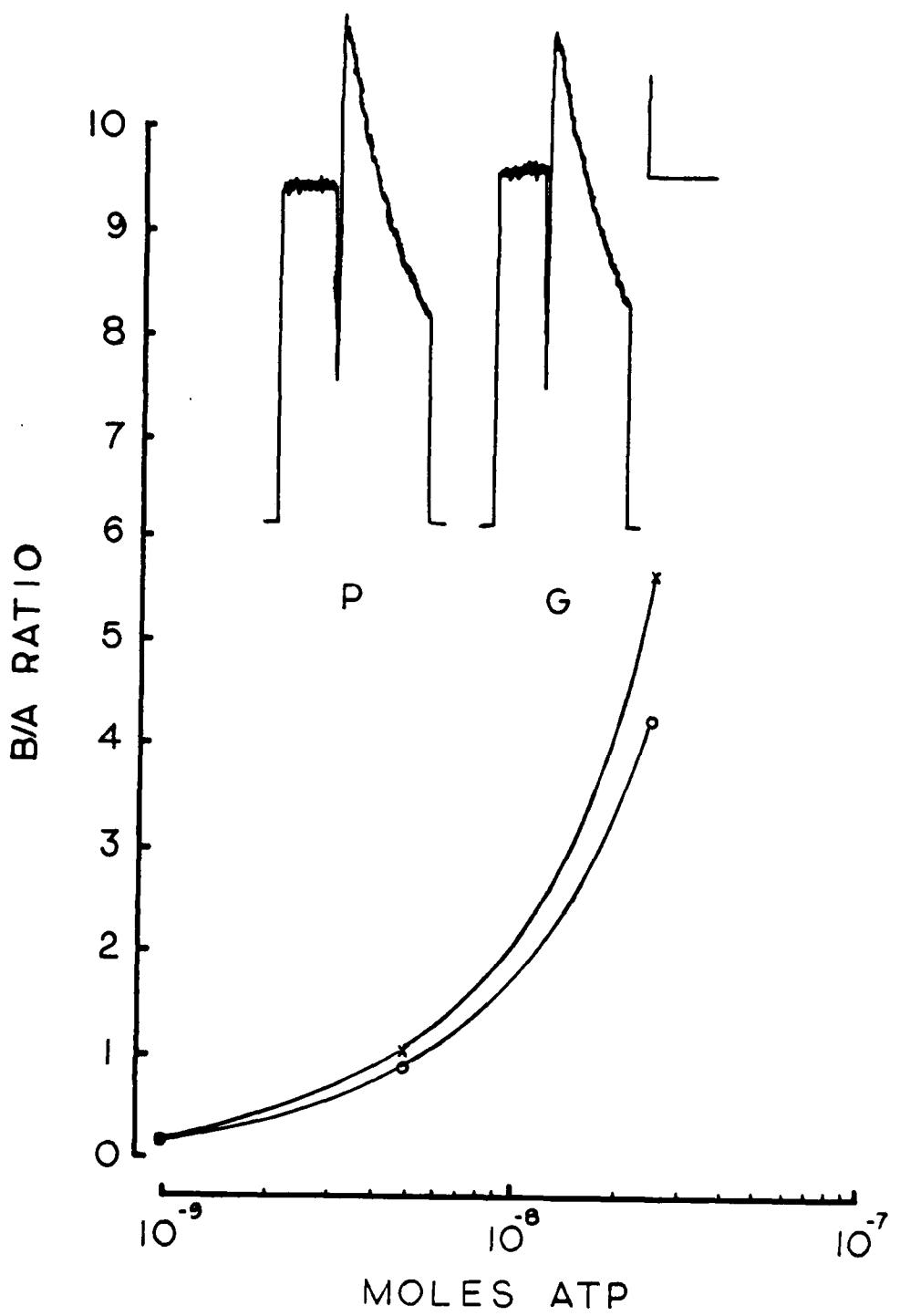


FIGURE 7

was less, and some quenching of the light signal was obvious.

Plastic cuvettes were subsequently used in all experiments.

Signal enhancement was made possible through the addition of synthetic luciferin to the reconstituted firefly lantern extract (FLE) (420). This is shown in Figure 8. The inset demonstrates that an assay of an ATP standard (25 nM) without excess luciferin (Figure 8i) exhibited an assay signal (component B) less than the background signal. In the presence of excess luciferin (Figure 8ii), the assay signal exceeded background emission. A comparison of the standard curves made with and without synthetic luciferin (Figure 8) shows that sensitivity was greater when synthetic luciferin was added. With the reactants present in excess, the amount of light emitted was only limited by the substance measured (ATP). Such enhancement with excess luciferin allowed quantification at nanomolar levels.

Use of the firefly bioluminescence assay also provided discrimination between various phosphorylated compounds. First, ATP standards (1 nM to 100 nM) that were assayed in a mixture of reconstituted firefly and synthetic luciferin, resulted in light

FIGURE 8
Effect of Synthetic Luciferin on the Firefly Bioassay.

Excess luciferin is necessary to preclude a rate limitation of the reaction (Figure 2) and to increase the sensitivity of the bioluminescence assay. The inset compares the light signals generated by the same ATP standard solution ($2.5 \times 10^{-8} M$).
i- without synthetic luciferin added; vertical bar, 10 mv.
ii- with 100 μ l synthetic luciferin (500 μ g/ml); vertical bar, 100 mv. Horizontal bar is 20 seconds. The standard curves were generated as in Figure 4. Sensitivity was enhanced with synthetic luciferin (o), allowing quantification in the nanomolar range, whereas almost a 10-fold higher concentration was needed to generate the same amount of light when synthetic luciferin was absent (x).

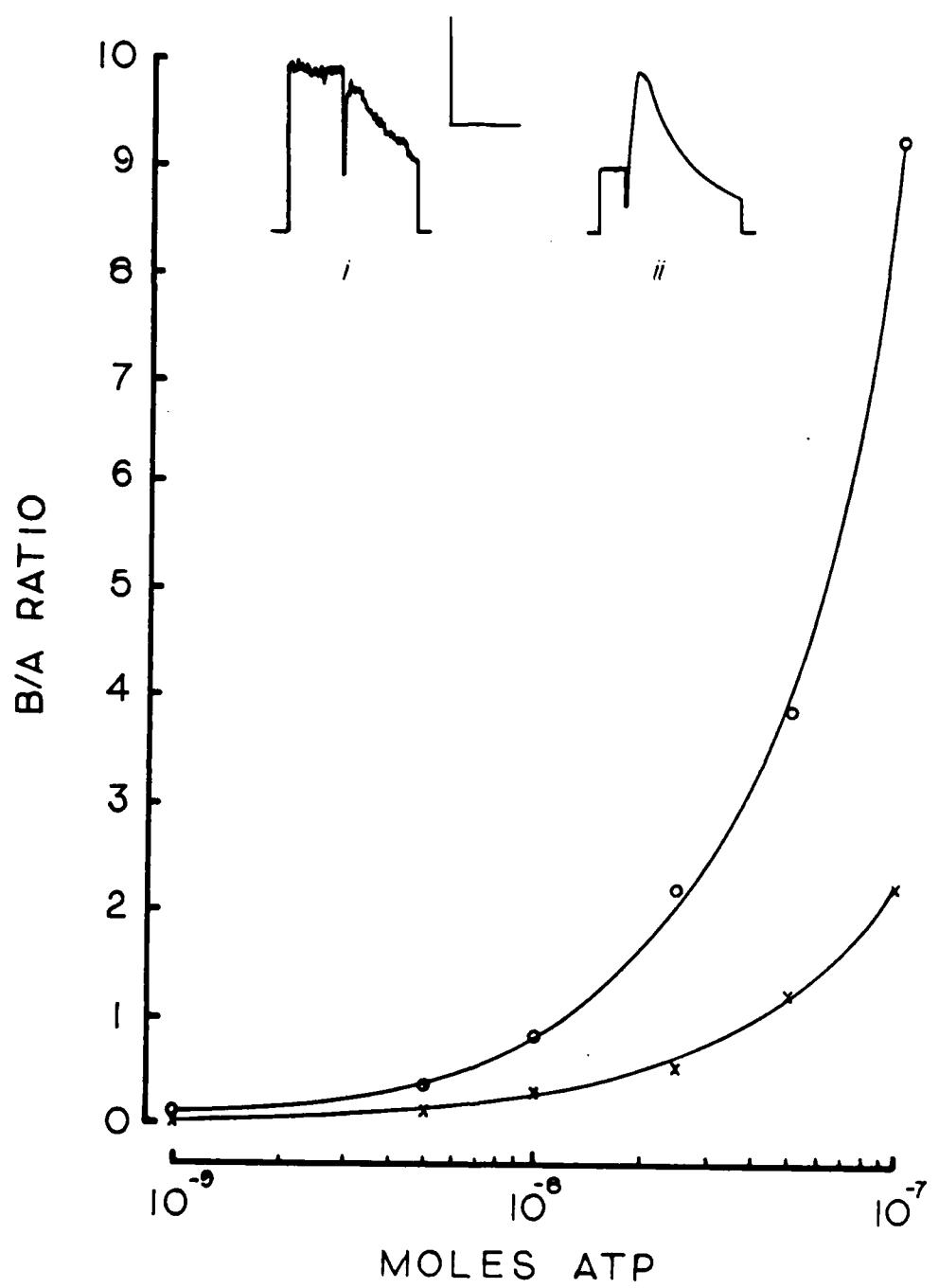


FIGURE 8

signals that exhibited three characteristics:

- 1) a dose-dependent light emission,
- 2) a rapid-rise increase in the light signal, and
- 3) an exponential decay of the light signal.

These are demonstrated in Figure 9.

Second, other adenine nucleotides or phosphorylated compounds demonstrated no such dose dependency or signal behavior at these concentrations. For instance, at an equivalent concentration (10 nM), ATP could be differentiated from ADP, AMP and adenosine (ADO) based on dose dependency (see Figures 10 and 11). Some small amount of ATP contamination of these latter three reagents as supplied by the company was apparent (see Figure 10).

Guanosine- and inosine-triphosphate exhibited signal decays that were dissimilar from the exponential decay of ATP. Additionally, their standard curves were significantly different from that of ATP (Figure 11). ADP, AMP and ADO caused no increase in the light signal with increasing concentration (Figure 11).

The final evidence substantiating ATP as the substance detected in the firefly bioassay involved the hydrolysis of ATP by apyrase (A-6132, Sigma). One unit of apyrase (11.7 units were used) produces 1 micromole of P_i per minute from ATP. This enzyme de-phosphorylates ATP to AMP by removing first the γ phosphate and then the β phosphate. Consequently, the assay signal was abolished. In samples containing red blood cells (see RESULTS) and ATP standards (not shown) the light signals generated with and without apyrase present were quantitatively distinct. It

AD-A173 871

EXTRACELLULAR ADENOSINE TRIPHOSPHATE ASSOCIATED WITH
AMPHIBIAN ERYTHROCYT. (U) AIR FORCE INST OF TECH
WRIGHT-PATTERSON AFB OH J P DIXON 1986

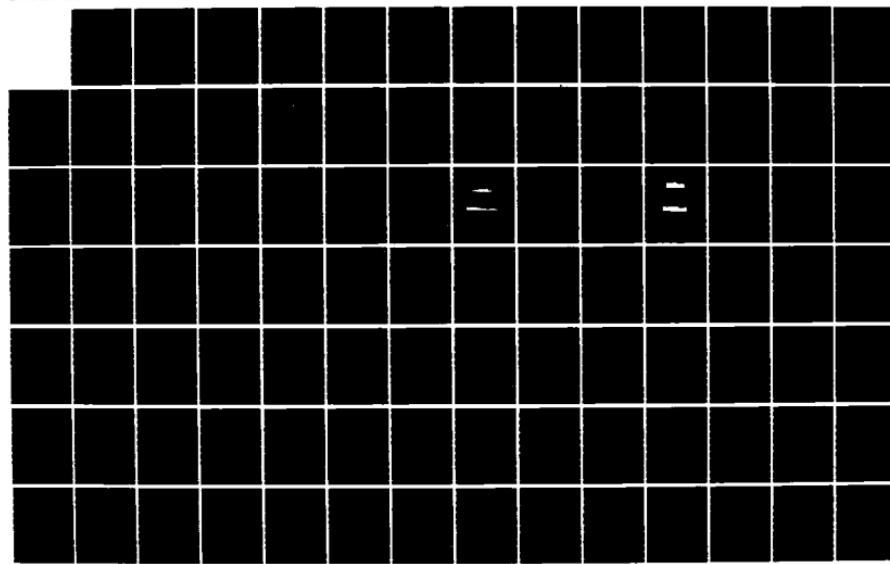
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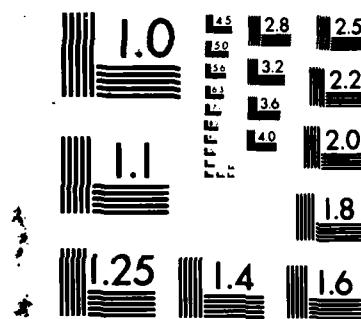
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MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

FIGURE 9
Recordings of Light Emissions Generated by ATP Standards.

Each signal was composed of the background signal, followed by the assay signal upon addition of 200 μ l of the standard solution to the firefly reaction mixture. Dose-dependent light emission is apparent. i- 1 nM, ii- 5 nM, iii- 10 nM, vertical bar, 10 mv, horizontal bar, 20 seconds; iv- 25 nM, v- 50 nM, vi- 100 nM, vertical bar, 100 mv, horizontal bar, 20 seconds.

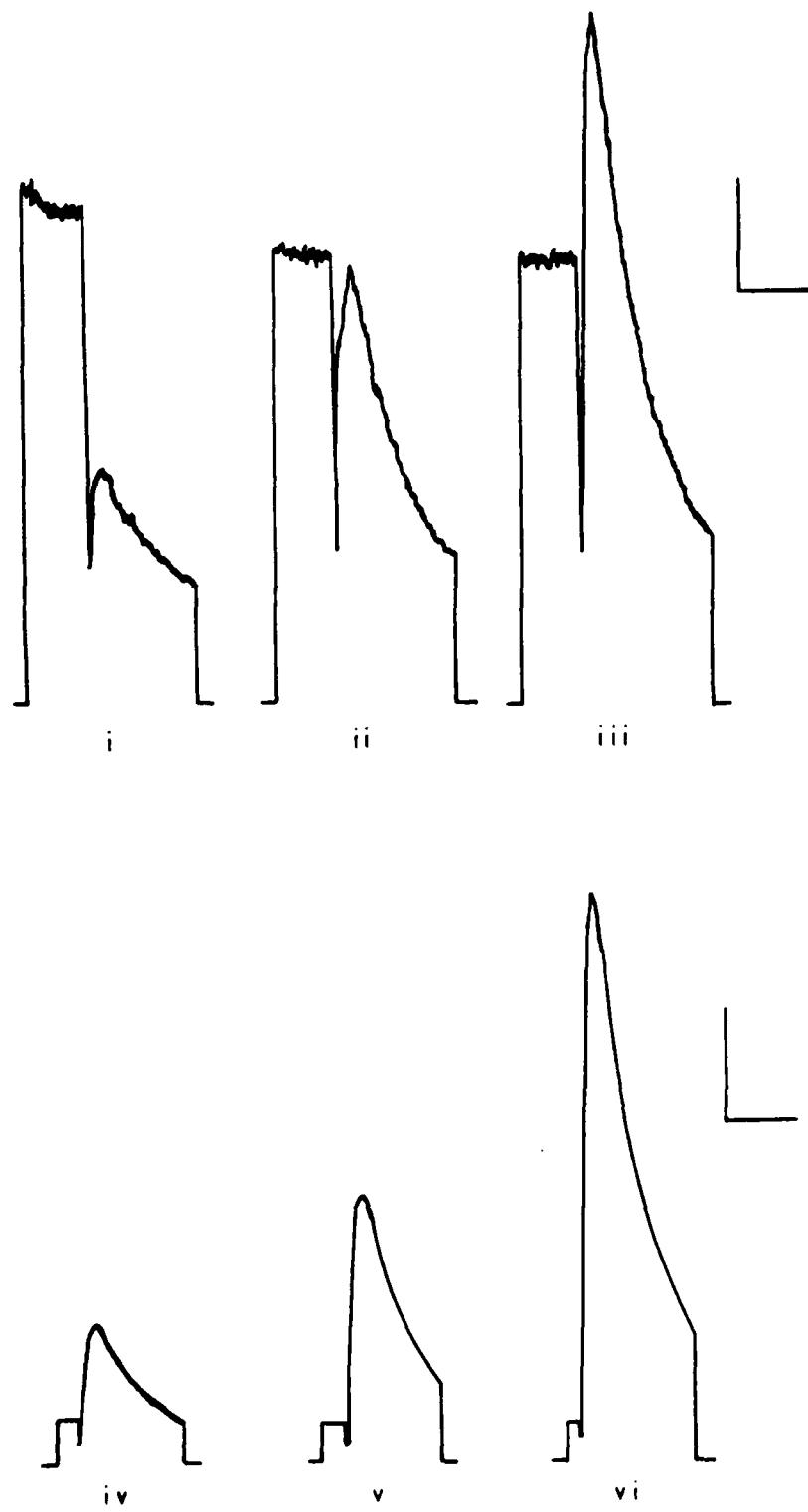


FIGURE 9

FIGURE 10
Comparison of Recordings of Light Signals
Produced by Various Purines.

Each signal was composed of the background light signal produced by the firefly reaction mixture alone, followed by the assay signal generated upon addition of 200 μ l of a standard solution (10 nM concentration each). ATP exhibited three characteristics: dose-dependent light emission, rapid-rise increase in the light signal, and an exponential decay of the light signal. A modicum of contamination by ATP was seen in the signals generated by ADP, AMP and ADO. The light signals generated by GTP and ITP were not characteristic of ATP. ATP- adenosine 5'-triphosphate, ADP- adenosine 5'-diphosphate, AMP- adenosine 5'-monophosphate, ADO- adenosine, GTP- guanosine 5'-triphosphate, ITP- inosine 5'-triphosphate.

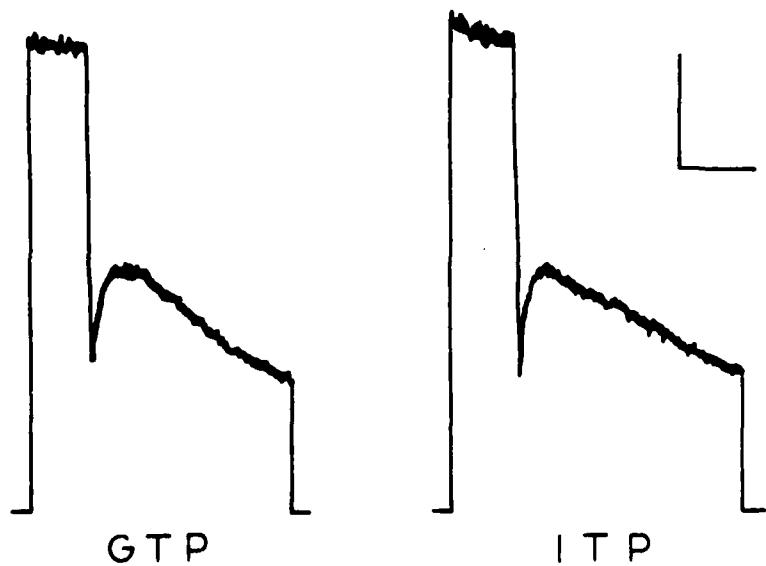
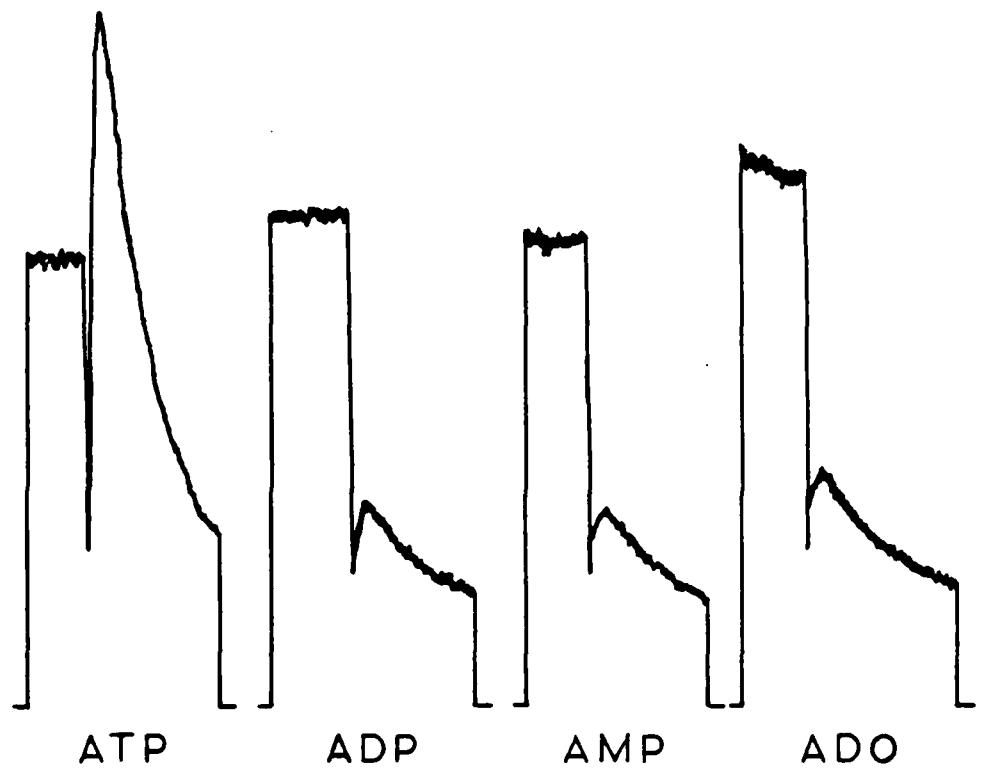


FIGURE 10

FIGURE 11
Standard Curves Generated with Various Purines.

The curves were generated as in Figure 4. (o)- ATP standard curve, (x)- standard curve representative of GTP or ITP, (•)- curve representative of ADP, AMP, or ADO. Synthetic luciferin was present in all.

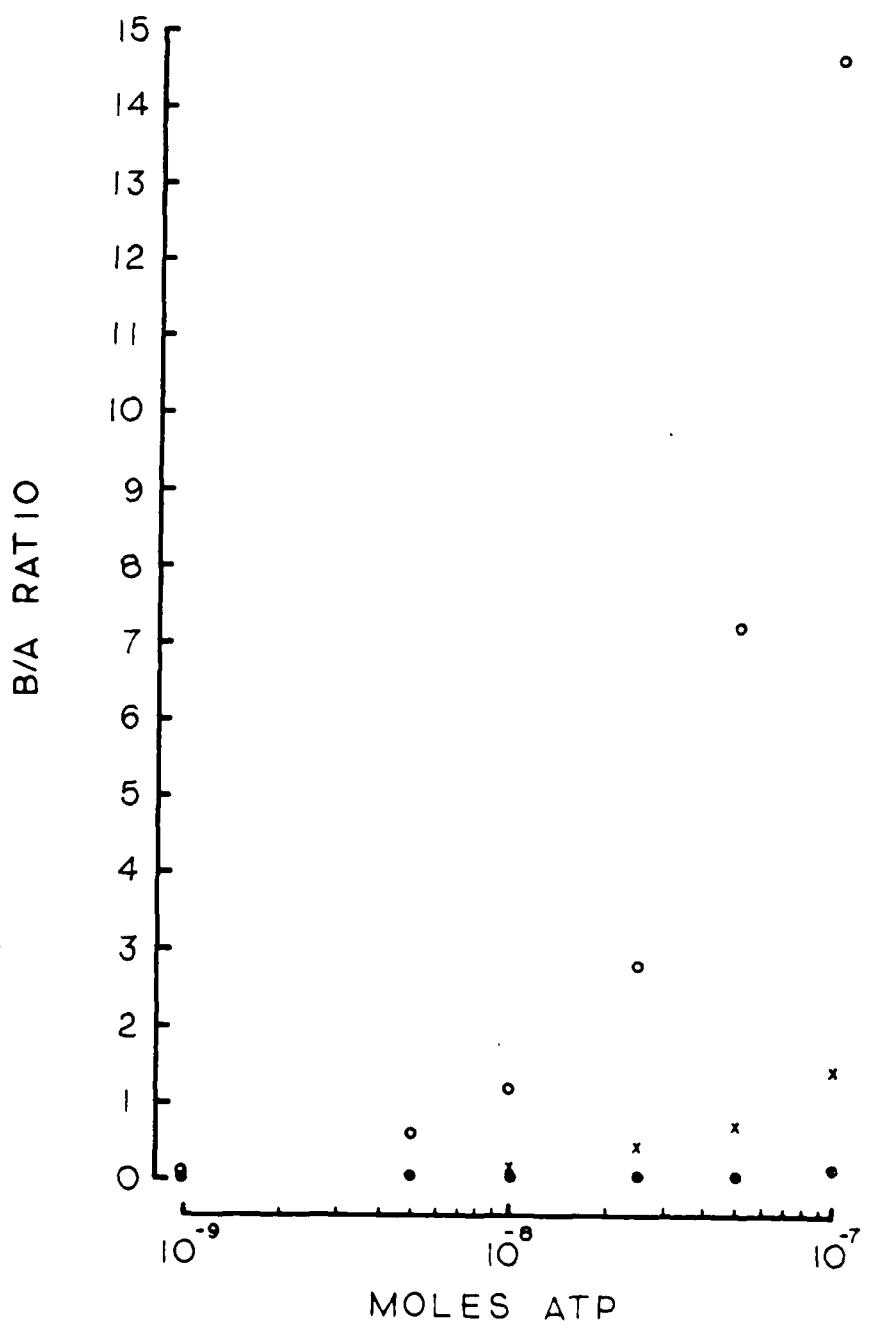


FIGURE 11

may be concluded from the aforementioned evidence that ATP was the substance that was quantitatively measured in the following experimental protocols.

Frog Heart Perfusion

Twenty-five frogs (Rana pipiens and Rana temporaria) were individually sacrificed by decapitation and pithing the spinal cord. During microscopic dissection using a Bausch & Lomb dissection microscope, the ventral surface of the frog was opened and the heart and accompanying blood vessels were surgically exposed. The pericardium was removed and the posterior vena cava cannulated with polyethylene tubing (Intramedic) through which a perfusion pressure of 90 mm H₂O was maintained with frog Ringer's solution. After exposing the aorta, the right branch was closed by suture and the left branch cannulated to the level of the conus arteriosus in order to sample the perfusate and monitor the heart rate and pulse pressure via a Gould Statham pressure transducer connected to a Grass Model 79D pen recorder. A functional representation of this apparatus is depicted in Figure 12.

In order to expose the heart to hypoxic conditions, the perfusate was made low in oxygen by bubbling with a 5% CO₂-95% N₂ gas mixture for 10 minutes. A hyperoxic perfusate was made by bubbling with a 5% CO₂-95% O₂ gas mixture. The effluent from the heart (measured in calibrated drops: 0.037 ml/drop) was

FIGURE 12
Diagram of Frog Heart Perfusion.

Dual syringes attached to a three-way stopcock, then to a 15-gauge needle and polyethylene tubing, were used to alternately perfuse frog Ringer's solution or a test solution (e.g., ATP standards, calcium blockers, P_2 receptor antagonist, etc.). Tubing from the aorta was attached to a pressure transducer, with effluent samples collected for ascertaining cardiac output and assaying for ATP release. The heart and the transducer were kept at the same vertical height. Tubing size- A: I.D.- 0.055 in., O.D.- 0.075 in.; B: I.D.- 0.86 mm, O.D.- 1.27 mm; C: I.D.- 0.023 in., O.D.- 0.038 in. Tubing lengths are shown in millimeters. PVC- posterior vena cava. Not to scale.

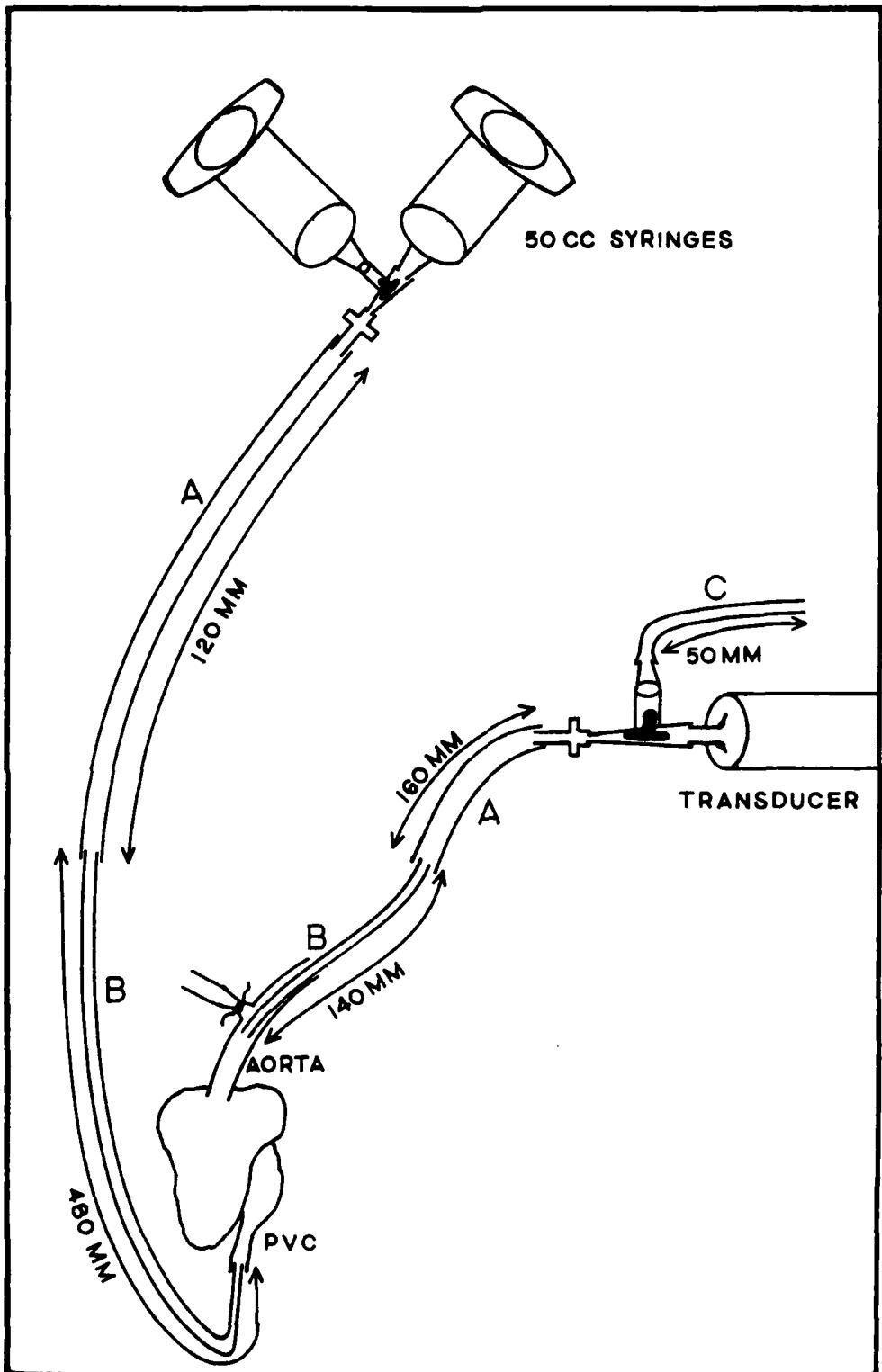


FIGURE 12

collected in plastic microvials and placed on ice. The time required to collect the samples was noted also. ATP was assayed in moles/liter and then divided by the wet weight of the ventricle (in grams) which had been dissected from the heart at the end of the experiment. This allowed determination of the ATP released (in pM/gm-min) from the frog myocardium during control conditions, and upon exposure to hypoxic and hyperoxic frog Ringer's solution. The pH, pO_2 and pCO_2 of the perfusate were determined using a blood gas analyzer (BMS MK 2 Blood Micro System, Radiometer, Copenhagen).

Erythrocyte Protocol

Leopard frogs (Rana pipiens) (NASCO or Charles D. Sullivan Co.) or salamanders (Amphiuma tridactylum) (Carolina Biological Supply, Burlington, NC) were used to provide blood samples for assaying ATP. Frogs were sacrificed as previously described. The right branch of the aorta was cannulated distally. Using frog Ringer's solution, a perfusion pressure of 200 mm H_2O was used to enhance venous return to the heart. The left bifurcation of the aorta was cannulated to the level of the conus arteriosus for blood sampling. Three to five drops of whole blood were allowed to empty into fifteen milliliters of frog Ringer's solution. Additional dilution of this RBC suspension was accomplished by pipetting 50-400 μ l into plastic microvials (Sarstedt) containing frog Ringer's solution to make a total volume of 1.4 ml. Samples were kept at room temperature.

Salamanders were anesthetized with tricaine beginning with a recommended dosage of 1:3000 and increasing the dosage as appropriate (367). A ventral incision was made to expose the heart and conus arteriosus. Blood was removed via hypodermic puncture of the conus arteriosus. Dilution of red cells was accomplished as described for frog RBC's.

Red blood cells were either allowed to settle by gravity in the plastic microvials for 50-60 minutes, or were maintained in mixed suspension on a rotator (Scientific Equipment Products) in which the microvials were inverted 20 times per minute. It was this "mixing" or "stirring", or the lack thereof, that proved crucial to documenting the efflux of ATP from these cells (see RESULTS).

An Aus Jena microscope was used to count the cells. The microscope eyepiece contained a graticule which was calibrated in microns for each power of objective used. Cell counts were made before and after ATP assays in an effort to monitor the degree of lysis, if any, in the isotonic frog Ringer's solution.

A hemocytometer (American Optical A-2440/B) was used to make the counts. The counting procedure was as follows. From a cell suspension, a sample was removed with plain micro-hematocrit capillary tubes (Curtin Matheson Scientific) and allowed to fill both counting chambers of the hemocytometer by capillary action. The 5 by 5 matrix in each counting chamber delimited the volume (0.1 μ l) that was counted. If cells touched the top and right

sides of the matrix, they were not counted. [Exception: Salamander red cells were so large ($> 70\mu$ in diameter) that all cells that overlapped the matrix borders were counted.] The average count of the two chambers (sum \div 2) was then divided by the volume counted (0.1 μ l).

Intracellular ATP concentration of frog red blood cells was determined by lysing the cells with a sonicator (Branson B-12), or by adding the detergent, digitonin. (See RESULTS for a description of the susceptibility of frog red cells to disruption by various concentrations of digitonin.) A 200 μ l volume of the lysed cells was then assayed as previously described.

A third method for calculating the intracellular ATP concentration was as follows. After injecting a 200 μ l sample of cells into the firefly-luciferin mixture and observing the assay signal generated by the extracellular ATP, digitonin (20 μ M final concentration) was injected to lyse the cells in front of the PMT. This gave a third estimate of intracellular ATP in frog red blood cells.

ATP Efflux Determination

Release of ATP from nucleated red blood cells was determined from the difference in the assay signals generated by the extracellular ATP (see RESULTS and Figure 36). The equation that was used to calculate ATP efflux in molecules per $\text{cm}^2\text{.second}$ is provided in Appendix III. ATP concentration was determined in moles/liter, corrected for the volume assayed (200 μ l) and

multiplied by Avogadro's Number. This was then divided by the time over which the flux occurred, corrected for seconds, and multiplied by the number of cells in the assay volume, which in turn was adjusted for surface area. The only assumption made was that the cell population was homogeneous with regard to cell surface area, i.e., surface area was assumed to be $422 \mu^2$ per cell (400). The calculations were made by computer (Sharp EL-5500III) using the program listed in Appendix IV. The software provided allows two consecutive flux calculations on the same sample if three different assays have been conducted. Efflux data presented throughout this work is based only upon the flux calculated with the first two consecutive assays of the same sample.

Drugs and Solutions

The physiological solution used to perfuse the frog heart in situ or to suspend the frog or salamander red blood cells was a modified Ringer's solution (106) maintained at room temperature (23°C) at a pH of 7.0. The constituents used (in mM) were as follows:

NaCl	109.00
KCl	3.80
NaHCO ₃	4.30
NaH ₂ PO ₄	0.22
CaCl ₂	5.00

Pharmacologic influences on alleged purinoceptors or other physiological mechanisms were studied using various agonists or antagonists. Table III is a list of all drugs and substances used

TABLE III
Drugs and Substances Used In This Study*

Adenosine	Quinidine
Adenosine 5'-triphosphate	Trifluoperazine
Adenosine 5'-diphosphate	Antazoline phosphate
Adenosine 5'-monophosphate	Lidocaine
Probenecid	Apyrase
Furosemide	Isatogen 151-3**
Theophylline	Tricaine
Lanthanum chloride (I)	Veratridine
Ouabain	Atractyloside
Manganese chloride	Dinitrophenol (DNP)
Ethylenediamine tetraacetic acid (EDTA)	Verapamil
Digitonin	
Pyridoxal 5'-phosphate (I)	
Trichloroacetic acid (TCA) (I)	
4-acetamido-4'-isothio-cyanostilbene-2,2'-disulfonic acid (SITS)	

(I) indicates that the drug or substance affected the firefly bioassay at the experimental concentration. See METHODS.

*All drugs were acquired from Sigma Chemical Company, St. Louis, MO, unless otherwise indicated.

**Source was Dr. Rudolph K. Winter. (See Figure 14 for more details.)

in this study. Each was dissolved in the physiological solution described previously. The concentration used is described in the RESULTS.

Firefly luminescence is an enzymatic reaction, which when used as a bioassay, may consequently be affected adversely by any substance included in the test solution. Each drug or substance used in this research to perfuse the heart or bathe the red cells was tested for its influence on the light emitted by the firefly bioassay. This was determined by making a duplicate standard ATP solution with the drug or substance also present in the solution at the same concentration used in the experiments. Any diminution in the B/A ratio as compared to an ATP standard without the drug was considered as evidence that the luciferin reaction (Figure 2) was adversely affected or inhibited. No conclusions were drawn from such data.

Figure 13 presents a comparison of the light signals generated by an ATP standard with and without a test substance, i.e., atracyloside in this instance. A similar comparison was made with all test substances to ensure that erroneous conclusions were not made. In Table I, those substances which affected the light emission from the firefly bioassay are annotated with an (I).

FIGURE 13
Comparison of Recordings of Light Signals Generated
by an ATP Standard Solution (10 nM) and
the Same Solution Containing Atractyloside.

Atractyloside (1 mM) did not affect light emission by the firefly, as seen by comparing the B/A ratio in "B" with the B/A ratio in A (no atractyloside). This same comparison was made with all drugs and substances added to the firefly reaction mixture, thus ensuring that the bioassay was not affected and that appropriate conclusions were drawn concerning the appearance of ATP in test samples.

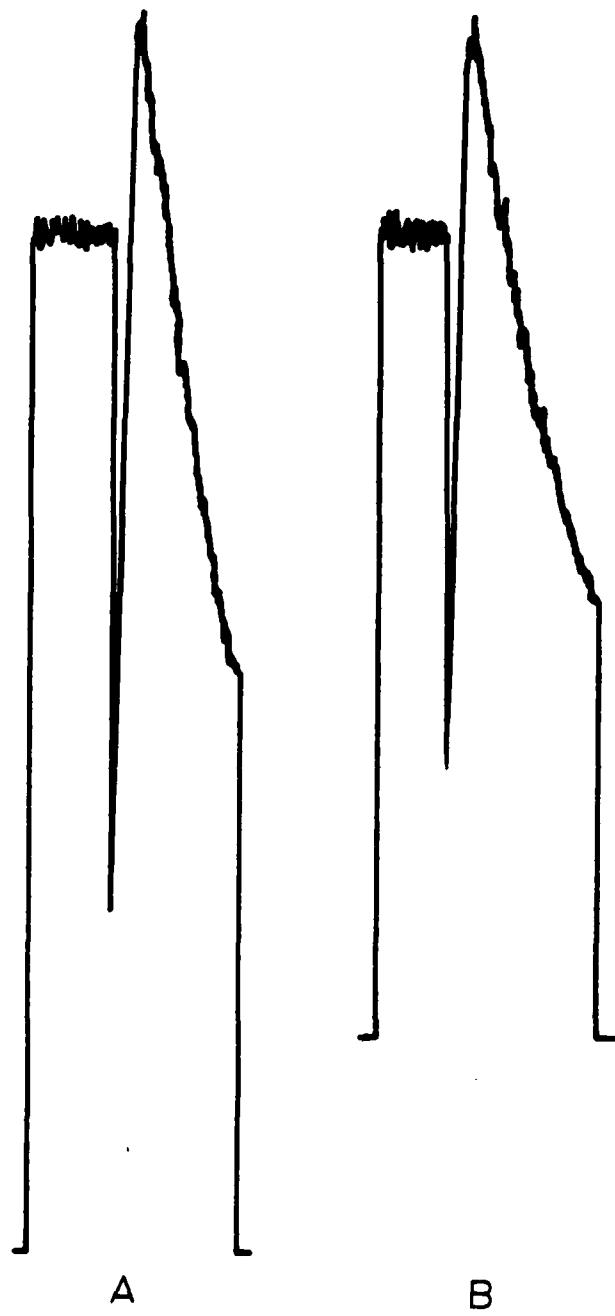
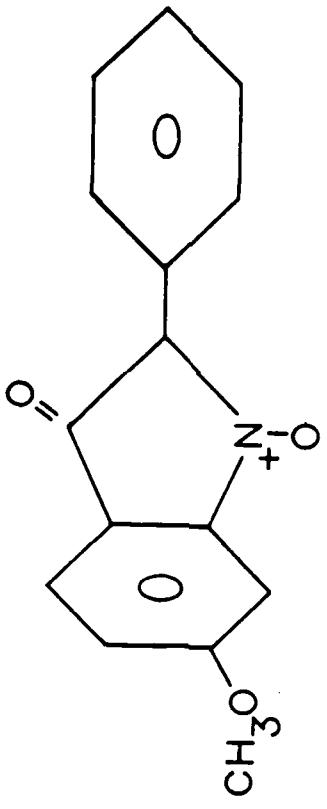


FIGURE 13

A new substance is introduced in this research. It was commonly called Isatogen 151-3 by Dr. R. Winter of Univ. of Mo., St. Louis. It is a proposed P₂ receptor antagonist, whose structure and molecular weight are described in Figure 14.

A Studentized t-test was used to statistically compare control and experimental tests. Data that were significant at a 95% level of confidence are identified by an asterisk.

FIGURE 14
A Proposed P₂ Purinoceptor Antagonist.
C₁₄H₁₀N₂O₃ HCl :
6-methoxy-2-(2' pyridyl)isatogen hydrochloride,
M.W. 290.5



RESULTS

ATP Release From Frog Myocardium

Hypoxic Insult

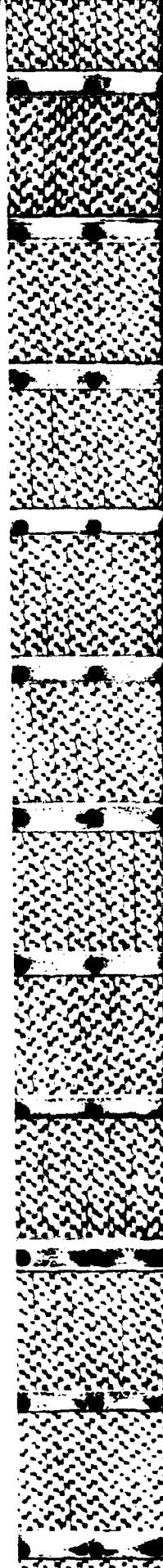
It was hypothesized that a hypoxic insult to the in situ frog heart would cause an increase in the appearance of ATP measured in the effluent samples taken from the aorta. As theorized by others (see HISTORICAL REVIEW) hypoxia may be a trigger in the release mechanism of ATP from tissue. An increase in the appearance of ATP was observed to occur when hypoxic frog Ringer's solution was perfused through the heart.

Figure 15a shows this increased appearance of ATP in one frog heart. The increased appearance was attributed to its release from the myocardium, and is presented as picomoles/liter per gram wet weight per minute. During the period of hypoxic insult, and for about four minutes thereafter, the ATP concentration was elevated. Only the last two samples (time: 7 1/2 - 10 minutes) taken during the testing period were near pre-exposure (control) levels found in the first sample.

The increase in appearance of ATP was accompanied by an increase in the systolic pressure and the cardiac output. Compared to pre-exposure values, the average systolic pressure of 15 hearts increased $31.3\% \pm 8.8$ (S.E.) during the 2 1/2 minutes when pO_2 was low (< 100 mm Hg). Similarly, cardiac output also increased, averaging $29.0\% \pm 9.4$ (S.E.).

FIGURE 15
ATP Released from Frog Myocardium During a) Perfusion
with Low Oxygen Solution or b) High Oxygen Solution.

Frog Ringer's solution with either low or high P_{O_2} was perfused through the heart during the time delineated by the arrow. Effluent was then collected and assayed. This figure from one experiment is representative of 7 of 15 hearts tested in this manner. During each 10 minute testing period six samples of the cardiac output were assayed for ATP. The ATP in the effluent during the control period (time: 0-1 minutes) was then compared to effluent collected during the period when P_{O_2} was altered (time: 1 1/2 - 4 minutes). (See METHODS for the calculation of ATP released.)



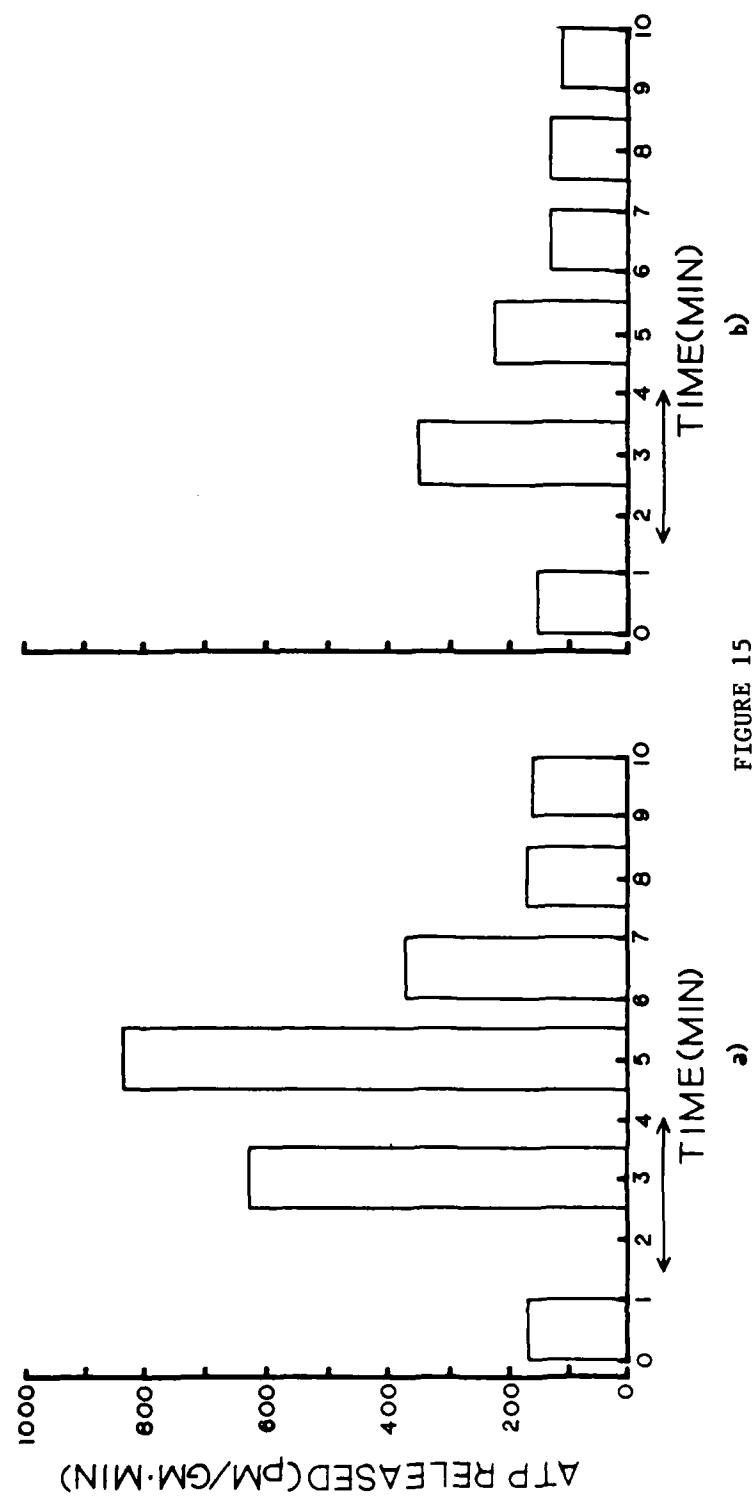
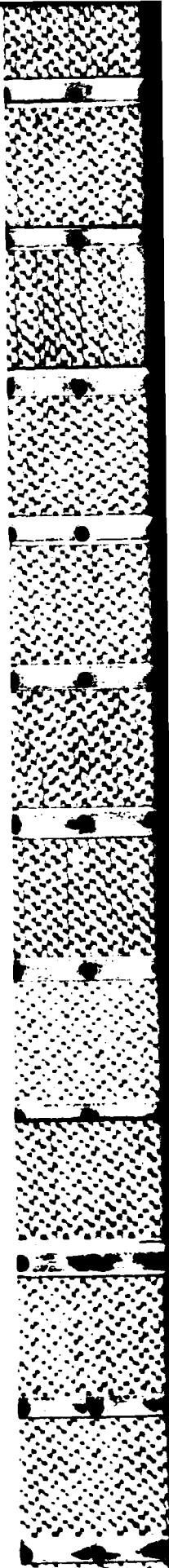


FIGURE 15



Hyperoxic Insult

Excess oxygen in a solution may not be luxury perfusion to tissue. Rather, it may be toxic. An identical protocol to that for the hypoxic exposure was used to measure the response of the in situ frog heart to high levels of pO_2 (>280 mm Hg). Were the high pO_2 excessively toxic, it was hypothesized that ATP appearance would change remarkably. Otherwise, little or no effect was expected.

Figure 15b shows the release of ATP in the same heart as shown in Figure 15a in response to hyperoxic frog Ringer's solution at least ten minutes after the hypoxic test period was completed. An increased release in the ATP occurred, though less than that observed in the previous protocol with low pO_2 as compared to the pre-exposure (control) level. Within 2 1/2 minutes following the end of the hyperoxic exposure, ATP was near control levels.

As with the hypoxia protocol, exposure to high pO_2 caused, in addition to an elevated release of ATP, an increase in the systolic pressure and the cardiac output. These parameters changed by an average of $25.9\% \pm 7.5$ (S.E.) and $31.3\% \pm 11.7$ (S.E.), respectively, for the 15 hearts studied.

To conclude a cause and effect relationship, the release of ATP during this protocol should have been consistent. In seven of

fifteen hearts studied, a similar pattern of ATP release, though with some variability, was observed like that presented in Figure 15. However, in the other eight hearts the appearance of ATP in the samples taken during the testing period was extremely variable and in no way consistent with the fact that the oxygen level was altered. Figure 16 shows this statistical comparison. No significant difference was observed in the amounts of ATP released when either hypoxic or hyperoxic frog Ringer's solution was perfused through the heart, as compared to the pre-exposure (control) levels of ATP.

The primary reason for this observation was that erythrocytes were found to be present in the effluent and overwhelmingly contributed to contamination of the ATP found in the samples. The ATP hypothesized to have been released from the frog myocardium, in some instances, originated from the red cells. This was only discovered when effluent samples were allowed to sit for 20-30 minutes, at which time a small red area appeared in the bottom of the plastic microvials containing the effluent. So few cells were present in each effluent sample that they could not be seen unless the cells had accumulated at the bottom. Documentation of this ATP associated with frog erythrocytes then became a priority (see below).

FIGURE 16
Comparison of ATP Released During Normoxic,
Hypoxic and Hyperoxic Perfusion of the Frog Myocardium.

In 15 hearts, the ATP released during the control period (normoxic perfusion) was compared to the period when pO_2 was altered (hypoxic or hyperoxic perfusion- see Figure 15). There were no significant differences ($\alpha = .05$) from control when oxygen levels were changed. Values indicate mean \pm S.E. (See METHODS for the calculation of ATP released.)

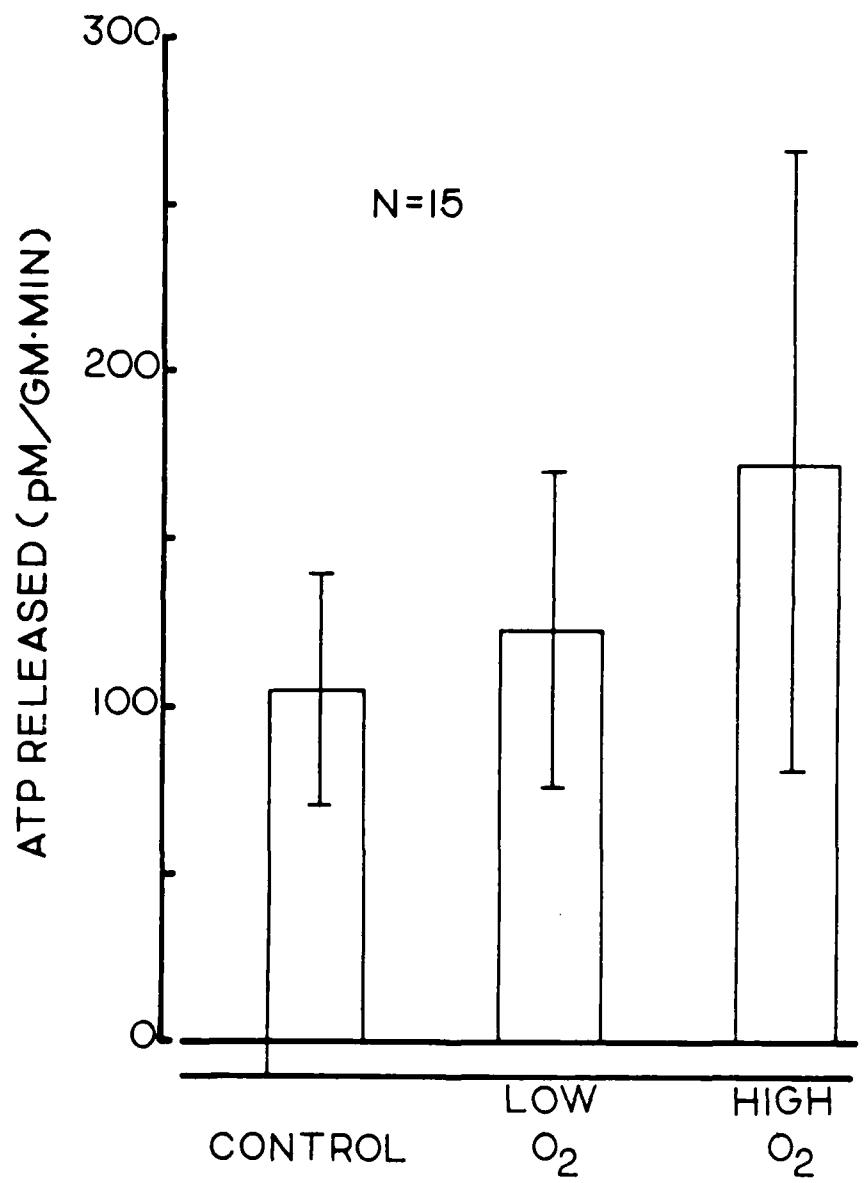


FIGURE 16

ATPase Activity of Frog Myocardium

Others (18,326) have studied the ability of the heart to degrade ATP that is perfused through the coronary circulation. Since the frog is without a coronary system and derives metabolic support from diffusion to and from the blood-filled heart chambers, it was of interest to study the degree of ATP hydrolysis which occurred when ATP solutions were perfused through the heart. This is shown in Figure 17.

Perfusing an ATP concentration of 1 nM through the heart resulted in no detectable hydrolysis. Rather, ATP was added to the perfusate and resulted in a positive difference between the effluent and perfusate. If higher concentrations of ATP were used, then ATP was degraded. The rate of hydrolysis had a range of over 1000 (mean of 5 hearts: 1.5×10^{13} to 5.1×10^{16} molecules/gm \cdot min), depending on the ATP concentration (10 nM to 10 μ M).

Calcium Mobilization:The Effect of Exogenous ATP on the Frog HeartATP and Calcium Blockers

The positive inotropic response of the frog heart to extracellular ATP has been described as triphasic (140,445). The initial, abrupt increase in contractility that is part of the triphasic response was studied in the in situ frog heart as an increase in the systolic pressure upon application of ATP. Figure

FIGURE 17
ATP Hydrolysis by Frog Myocardium.

Standard solutions of ATP (shown here as whole numbers: 5,6,7,8, and 9 indicate the negative Log of the molar concentration) were perfused through frog hearts ($n=5$) and the effluent collected and assayed for ATP. The average rate of hydrolysis (in molecules per gm min) was calculated using the equation in APPENDIX V, utilizing the difference in ATP concentration between the effluent and perfusate, and the cardiac output and wet weight of the frog ventricle. A quantity above the midline indicates a positive difference between the effluent and the perfusate, whereas below the midline indicates a negative difference (ATP hydrolysis).

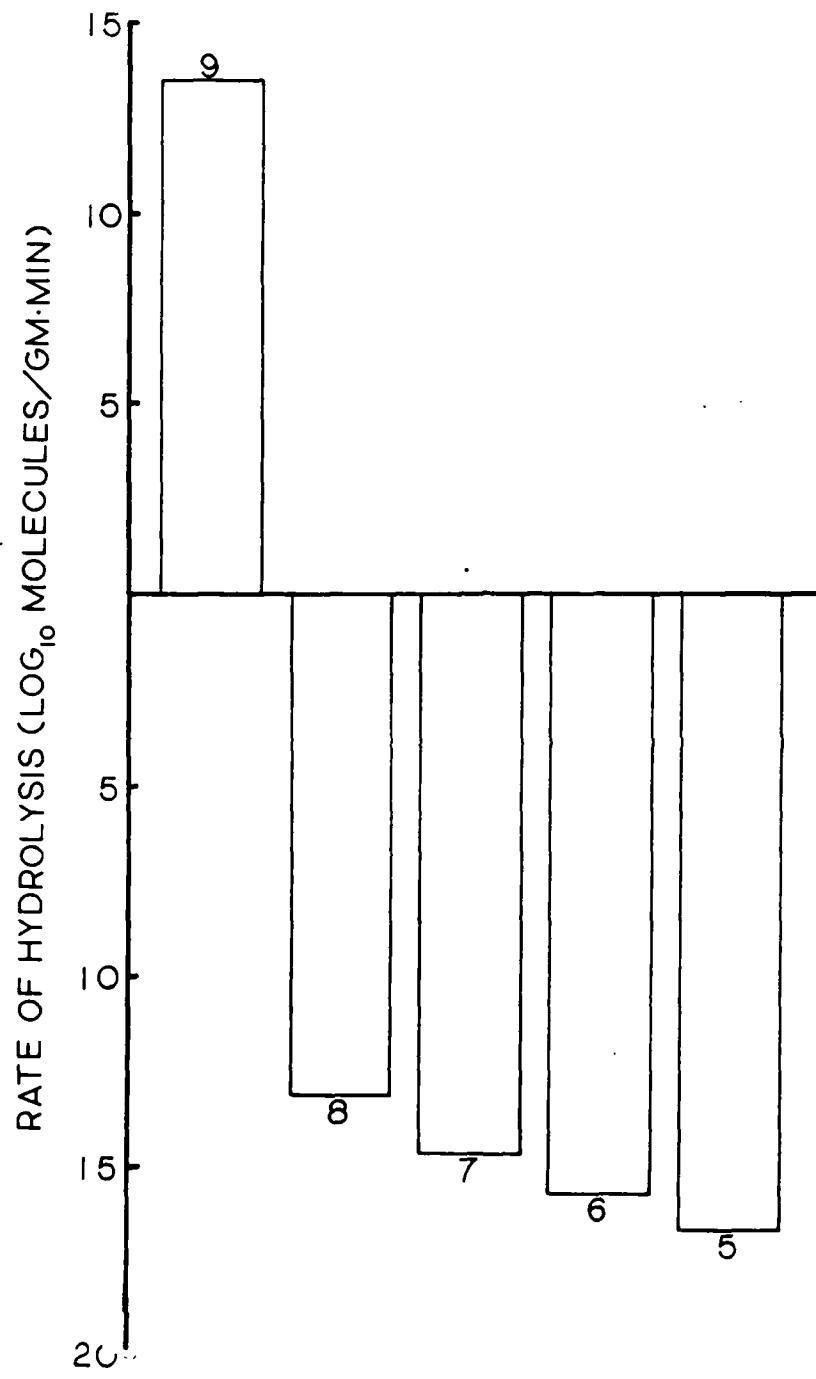


FIGURE 17

FIGURE 18
Systolic Pressure Changes Following Perfusion of
Exogenous ATP Through Frog Myocardium.

Five different concentrations of ATP were perfused through the hearts (n = number of hearts) and the maximum percent change in systolic pressure at each concentration was ascertained. A dose-dependent increase in systolic pressure is apparent. Values indicate the mean \pm S.E.

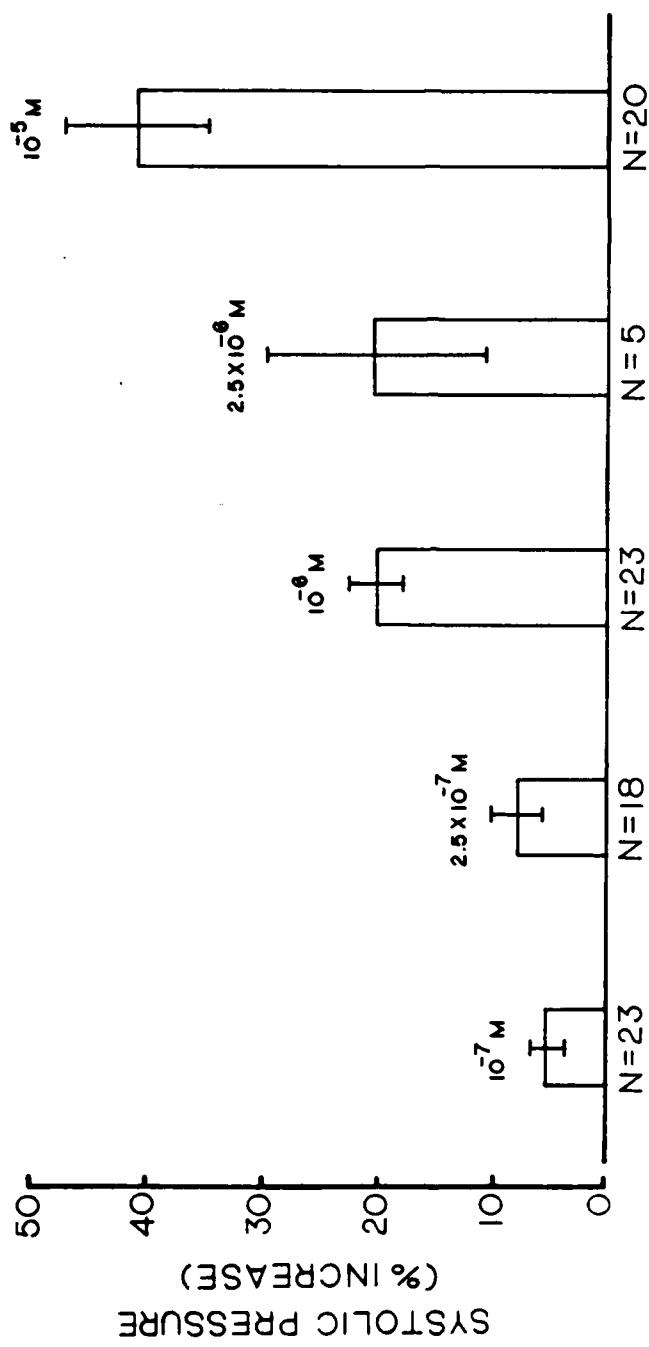


FIGURE 18

18 demonstrates that response of the myocardium to P_{2X} receptor activation was dose-dependent. ATP at successively higher doses produced graded inotropic responses that could be measured as a transient increase in the systolic pressure. This transient rise in systolic pressure corresponded to the initial phase of the triphasic response described by Flitney and Singh (140). This is seen in Figure 19a.

The origin of the calcium mobilized in response to ATP has been hypothesized to be the sarcoplasmic reticulum (SR) (320). However, experience with two calcium blockers, manganese and verapamil, suggests that some of the calcium originated from a non-cytoplasmic source. When frog hearts were continually exposed to these calcium blockers, the response, as expected, was a progressive decrease in the pulse pressure. Contractility diminished quickly as calcium was displaced from its external binding sites and prevented from replenishing the contractile calcium inside the muscle cells. If, however, a calcium blocker (e.g., manganese at 1.5 mM) was used sparingly for a brief period of time prior to the ATP exposure, the inotropic response to ATP by the heart was enhanced.

Figure 19b shows that the transient rise in the systolic pressure was significantly increased by the sequential perfusion of manganese and ATP (10 μ M) when compared to ATP alone.

FIGURE 19

The Effect on Systolic Pressure of Perfusing the Frog Heart with Exogenous ATP Alone or Immediately Following $MnCl_2$.

The traces indicate the pulse pressure at 90 mm H_2O perfusion pressure. a) Control- 85 seconds after the start of perfusion of 10 μM ATP (single arrow), systolic pressure peaked as the P_{2X} receptor mobilized calcium. The inotropic response as described by Flitney and Singh (140) is not shown here in its entirety. Double arrows- re-perfusion with frog Ringer's solution. b) Single arrow- start of perfusion with $MnCl_2$ (1.5 mM); Double arrows- start of perfusion with ATP (10 μM), followed 87 seconds later by an enhanced inotropic response. The delay between the arrow and the recorded response is due to the transit time of the perfusate through the tubing to the heart (see Figure 12). Time trace: tics indicate 5-second intervals. Vertical scale: 1 cm = 7.5 mm H_2O .

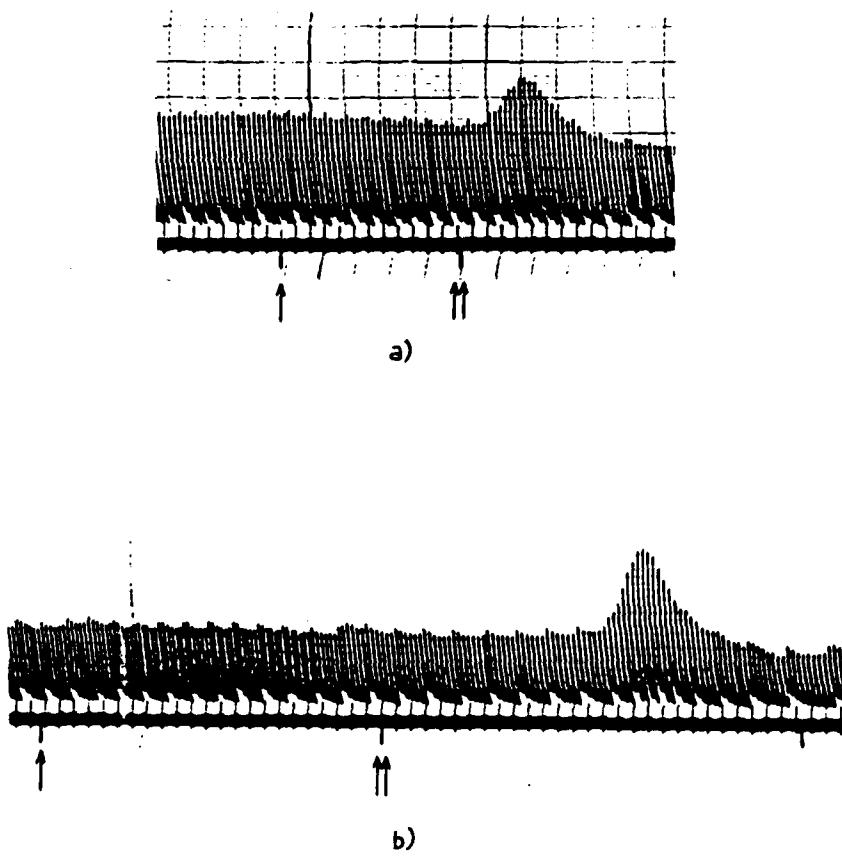


FIGURE 19

Manganese displaced calcium from its binding sites, an event that increased the calcium available to be mobilized by ATP. An increase in the inotropic response of the heart resulted.

This increased inotropism was not peculiar to manganese. Figure 20 demonstrates, using the same technique, that verapamil (1 μ M) followed in tandem by ATP (10 μ M) also increased the pool of calcium that was mobilized by ATP interaction with its receptor (Figure 20b). A significant elevation in systolic pressure was observed over that seen when ATP alone was used (Figure 20a).

Each of these protocols utilizing the calcium blockers, manganese and verapamil, provided a statistical basis for concluding that at least some of the calcium mobilized by ATP originates in the extracellular space. Figures 21 and 22 show that the percent increase in systolic pressure, which is a measure of the increased contractility caused by ATP, was not an indiscriminate effect. The transient increase in contractility was due to an enhanced availability of calcium.

ATP and a Proposed Purinergic Antagonist

In an attempt to block the action of ATP on its excitatory receptor in the heart, a new substance was tested. This substance, called Isatogen 151-3, is a pyridylisatogen derivative (see METHODS). It was perfused through the heart in frog Ringer's

FIGURE 20

The Effect on Systolic Pressure of Perfusing the Frog Heart with Exogenous ATP Alone or Immediately Following Verapamil.

The traces indicate the pulse pressure at 90 mm H₂O perfusion pressure. a) Control- 35 seconds after the start of perfusion of 10 μ M ATP (single arrow), systolic pressure peaked in response to calcium mobilization by the P₂X purinoceptor. Double arrows- re-perfusion with frog Ringer's solution. b) Single arrow- start of perfusion with verapamil (1 μ M); Double arrows- start of perfusion with ATP (10 μ M), followed 38 seconds later by an enhanced inotropic response. Time trace: tics indicate 5-second intervals. Vertical scale: 1 cm = 7.32 mm H₂O.

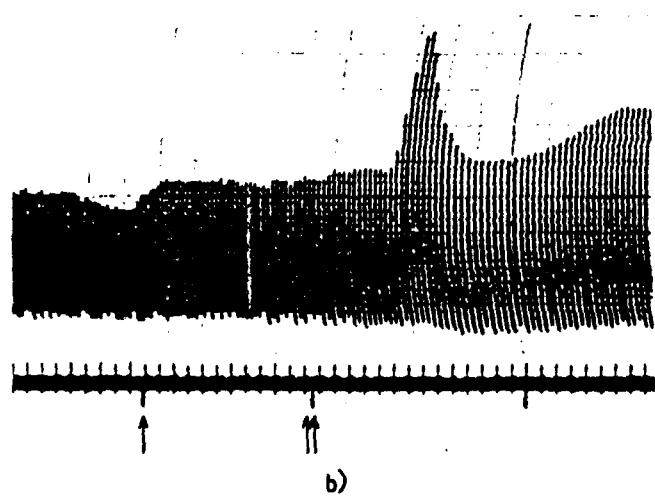
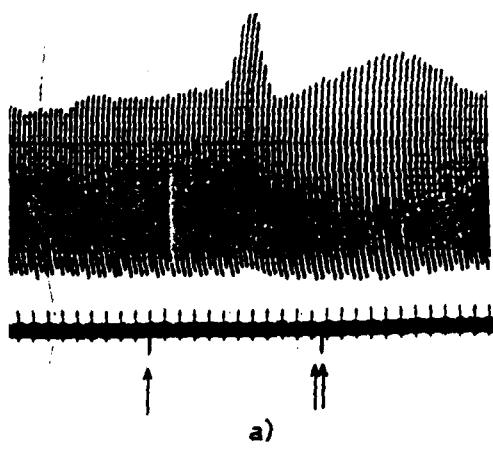


FIGURE 20

FIGURE 21
Comparison of Systolic Pressure Changes Caused
by ATP With and Without Manganese Present.

Maximum percent increase in systolic pressure following perfusion with exogenous ATP alone or in tandem with manganese, a calcium blocker. The experimental procedure was the same as in Figure 20, here comparing the maximum % increase in systolic pressure under control conditions (10 μ M ATP alone) and during ATP perfusion after 45-60 seconds of exposure to 1.5 mM MnCl₂ (* significant at $\alpha = .05$). Values indicate mean \pm S.E.

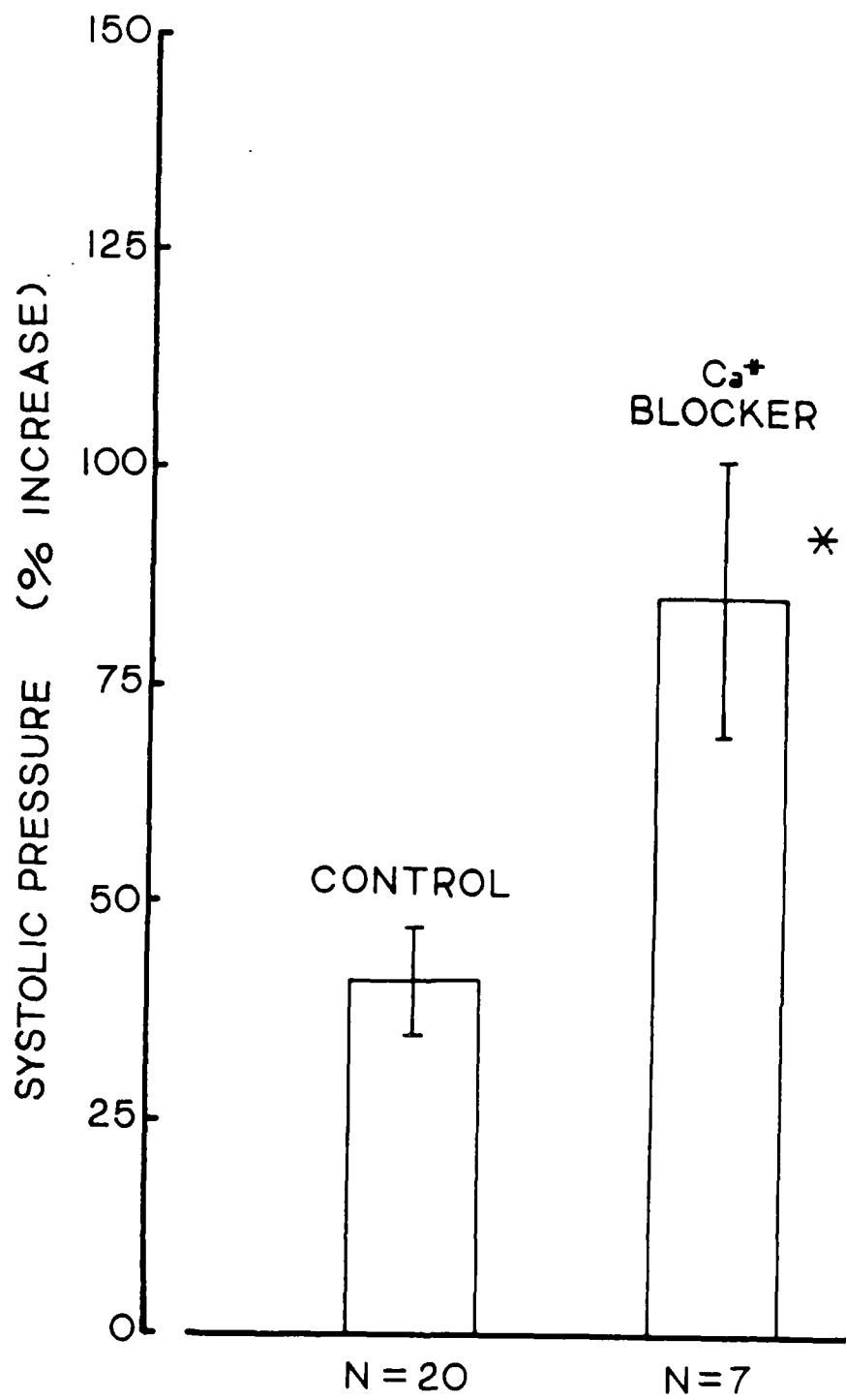


FIGURE 21

FIGURE 22
Comparison of Systolic Pressure Changes Caused
by ATP With and Without Verapamil Present.

Maximum percent increase in systolic pressure following perfusion with exogenous ATP alone or in tandem with verapamil, a calcium blocker. The experimental procedure was the same as in Figure 20, here comparing the maximum % increase in systolic pressure under control conditions (10 μ M ATP alone) and during ATP perfusion after 45-60 seconds of exposure to 1 μ M verapamil (* significant at $\alpha = .05$). Values indicate mean \pm S.E.

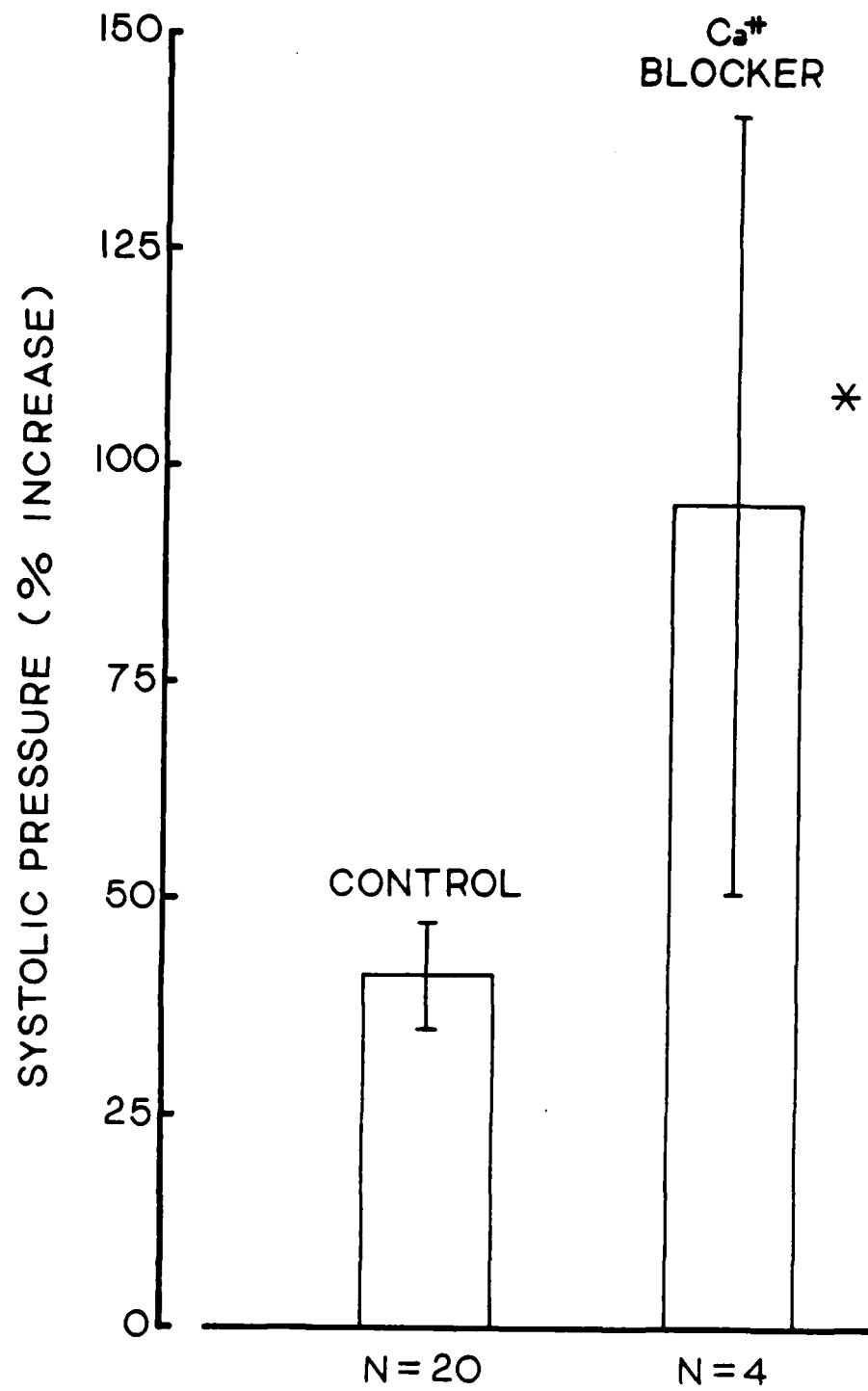


FIGURE 22

solution at a concentration of 0.5 M for 10-15 minutes before ATP (10 μ M) was used. This proposed P_2 antagonist decreased the systolic pressure response to ATP (Figure 23). However, with so few observations, additional experiments are required to unequivocally determine whether or not Isatogen 151-3 effectively blocked the positive inotropic effect of ATP.

ATP of Amphibian Erythrocytes

Extracellular ATP Associated With Gravity-Settled Cells

Experience with the effluent coming from the frog heart gave cause for concern. Although the effluent samples appeared transparent, cells were observed to form a noticeable red spot in the bottom of the collecting microvial when the samples were left untouched for over 20 minutes. The cells were obviously of such mass that they could settle out by gravity. A count of the cells in the effluent sample when it was thoroughly mixed showed that the allegedly "transparent" effluent actually contained 50-500 cells/ μ l. Additionally, ATP assays of these samples demonstrated that ATP was differentially distributed in the sample.

Assays of the top of the effluent, or near the bottom around or including the red cells, showed that ATP was not equally dispersed throughout the solution. Also, the ATP concentration of an effluent sample did not remain stable. Sometimes (usually) it

FIGURE 23
Effect of a Proposed P₂X Blocker on
Systolic Pressure Changes Caused by ATP.

The proposed P₂X blocker, Isatogen 151-3 at a concentration of 0.5 μ M, was perfused through the frog heart for 10-15 minutes. ATP (10 μ M) was then perfused through the heart and the percent change in systolic pressure was measured. Isatogen 151-3 appeared to antagonize the excitatory purinoceptor, though the results were not statistically significant at an $\alpha = .05$. Values indicate mean \pm S.E.

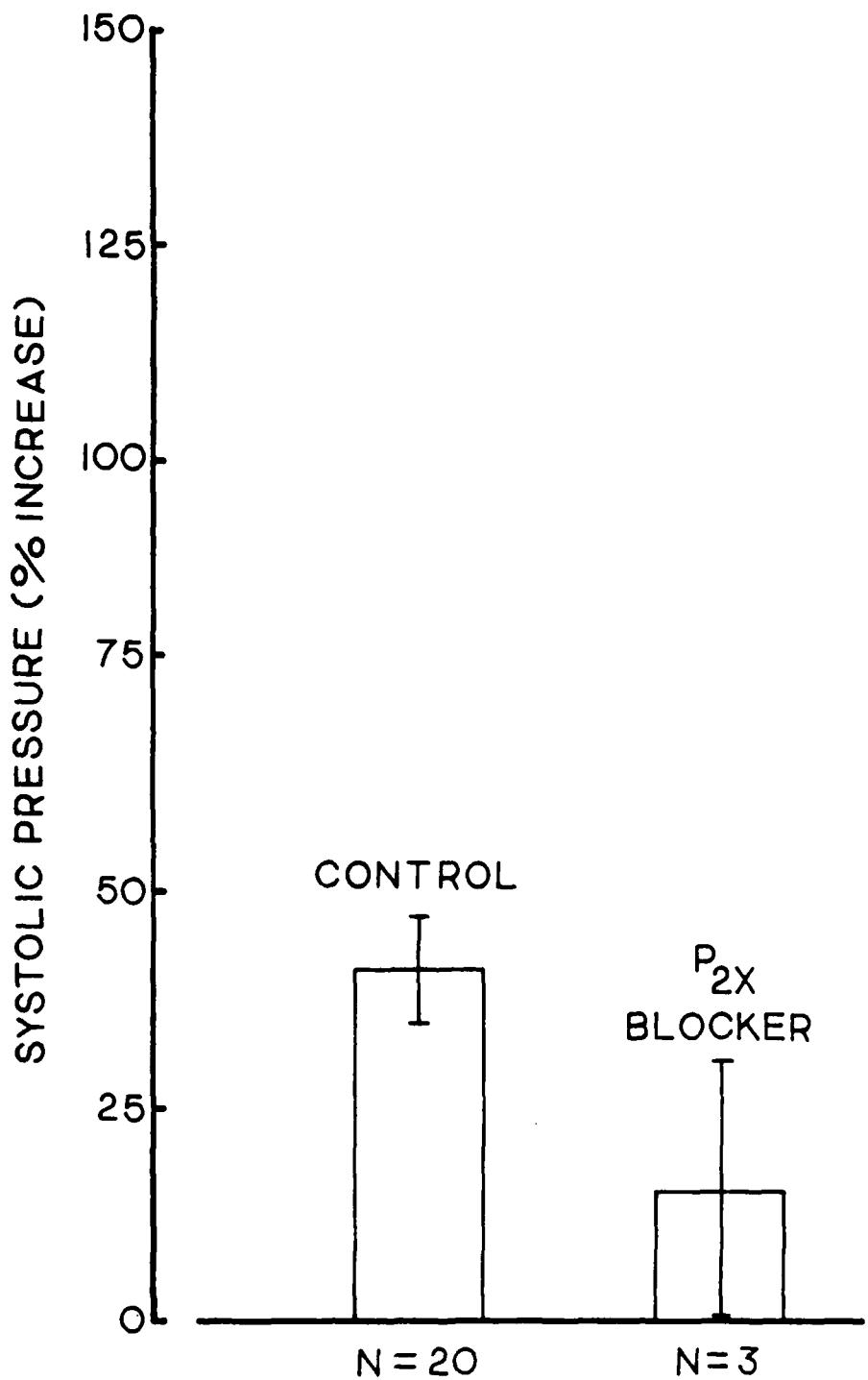


FIGURE 23

increased, but sometimes it diminished. This meant that until the problem of ATP contamination was solved, study of ATP release from the frog myocardium was not likely to be a productive one.

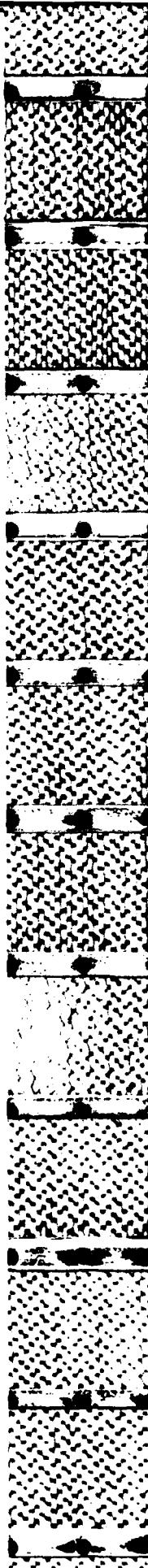
Whole blood was harvested and diluted into plastic microvials with frog Ringer's solution. The samples were deliberately diluted so that they closely resembled heart effluent samples in transparency (i.e., few or no cells could be observed) and in count (80-1200 cells/ μ l). When the cells were allowed to settle by gravity, a layering of the ATP in the supernatant over the cells was found. This is shown in Figure 24.

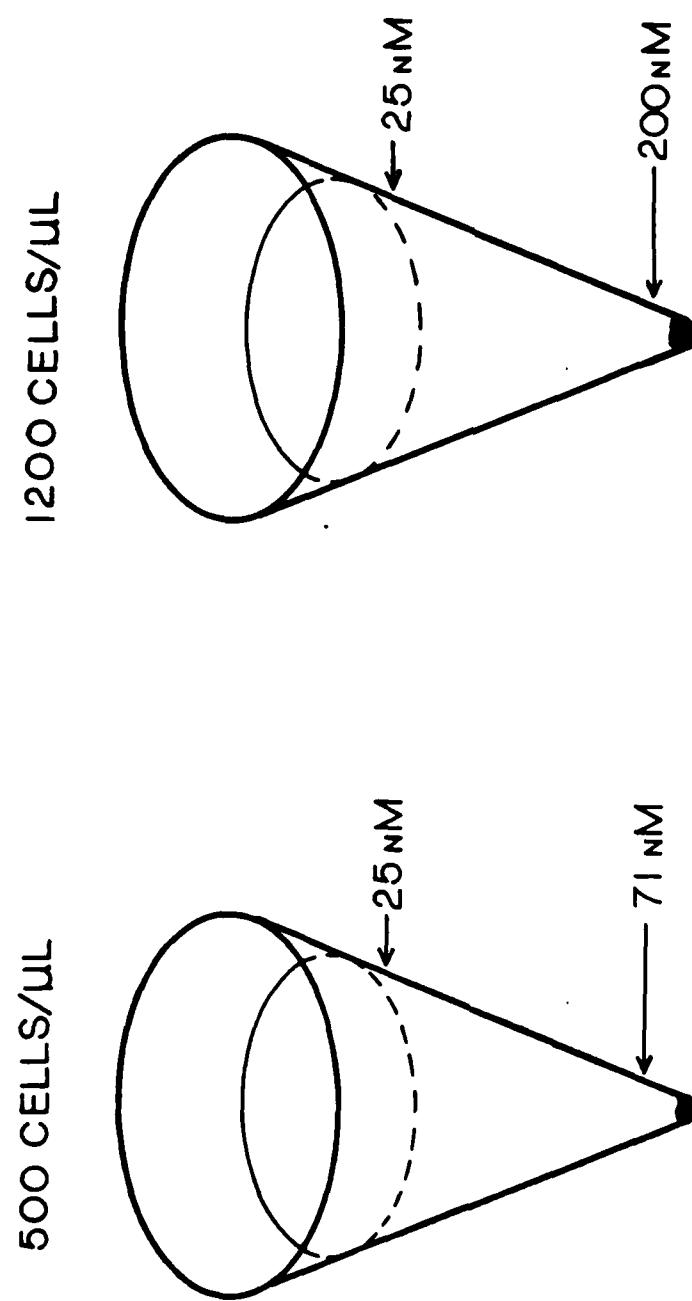
When the supernatant fluid was assayed near the surface, the ATP concentration was almost identical for all the diluted cell samples from the same frog. Thus, the ATP concentration at the top of the supernatant was independent of the cell count.

Contrary to expectations, the ATP was not observed to be equally dispersed throughout the solution. When the supernatant fluid was assayed near the cells, the concentration far exceeded that at the surface. Based on cell counts made when the cells were thoroughly mixed, the more cells that were settled to the bottom, the greater the ATP concentration near them. Thus, the ATP level near the cells was cell-dependent.

FIGURE 24
ATP Layering Associated with Gravity-Settled Cells.

Cell samples (1.4 ml total volume of whole blood diluted approximately 1000-fold) allowed to settle by gravity for 60 minutes exhibited increased ATP levels. Near (2 mm above) the cells, the ATP concentration was dependent on the cell count. ATP concentration at the surface of the supernatant was independent of the cell count.

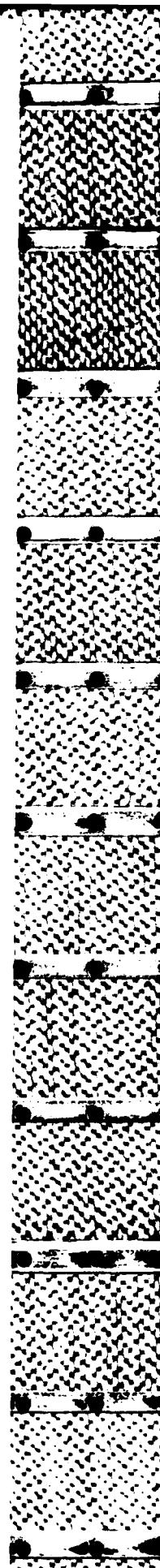




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ATP LAYERING

FIGURE 24



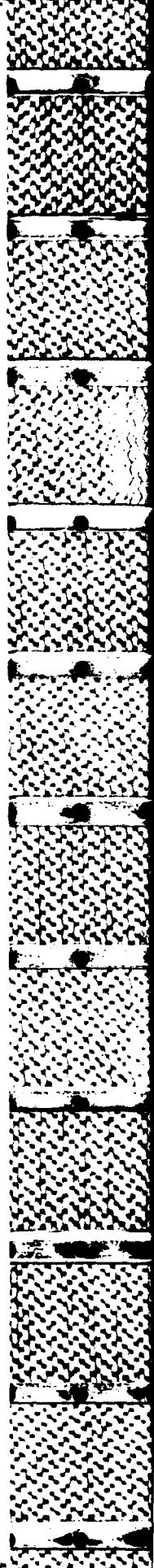
This observation was further verified when cell counts were plotted versus the ATP concentration of the supernatant fluid or the gently mixed cell suspensions. Figure 25 shows that the ATP concentration at the surface of the supernatant was always low, whereas the ATP associated with the settled cells became greater and was a function of the number of cells present.

The concentration of ATP around the settled cells was significantly greater, but not as great as if the cells were lysed. Compare the assay levels in Figure 25 with those seen in Figure 26. In the latter figure, cells lysed by sonication resulted in ATP levels that were, on a per-cell basis, over 10 orders of magnitude greater than the ATP associated with the outside of the cells. The ATP layering effect was a consequence of ATP being released from the cells, and should not be confused with intracellular ATP from cells lysed during the protocol procedure.

ATP appearance above gravity-settled cells contrasted with a relatively stable level of ATP seen when the cells were continuously stirred by maintaining the microvials on a tube rotator. This is seen in Figure 27. Cells that were stirred never released ATP at a rate such that a noticeable increase in the concentration could be documented.

FIGURE 25
Extracellular ATP Concentration in Cell
Samples Before and After Gentle Dispersion.

The supernatant fluid (o) was consistently low in ATP when cells were allowed to settle by gravity. However, gentle dispersion (x) of the cells throughout the medium also dispersed the ATP associated with the cells, increasing the extracellular ATP concentration. In general, the greater the cell count, the greater the concentration of extracellular ATP.



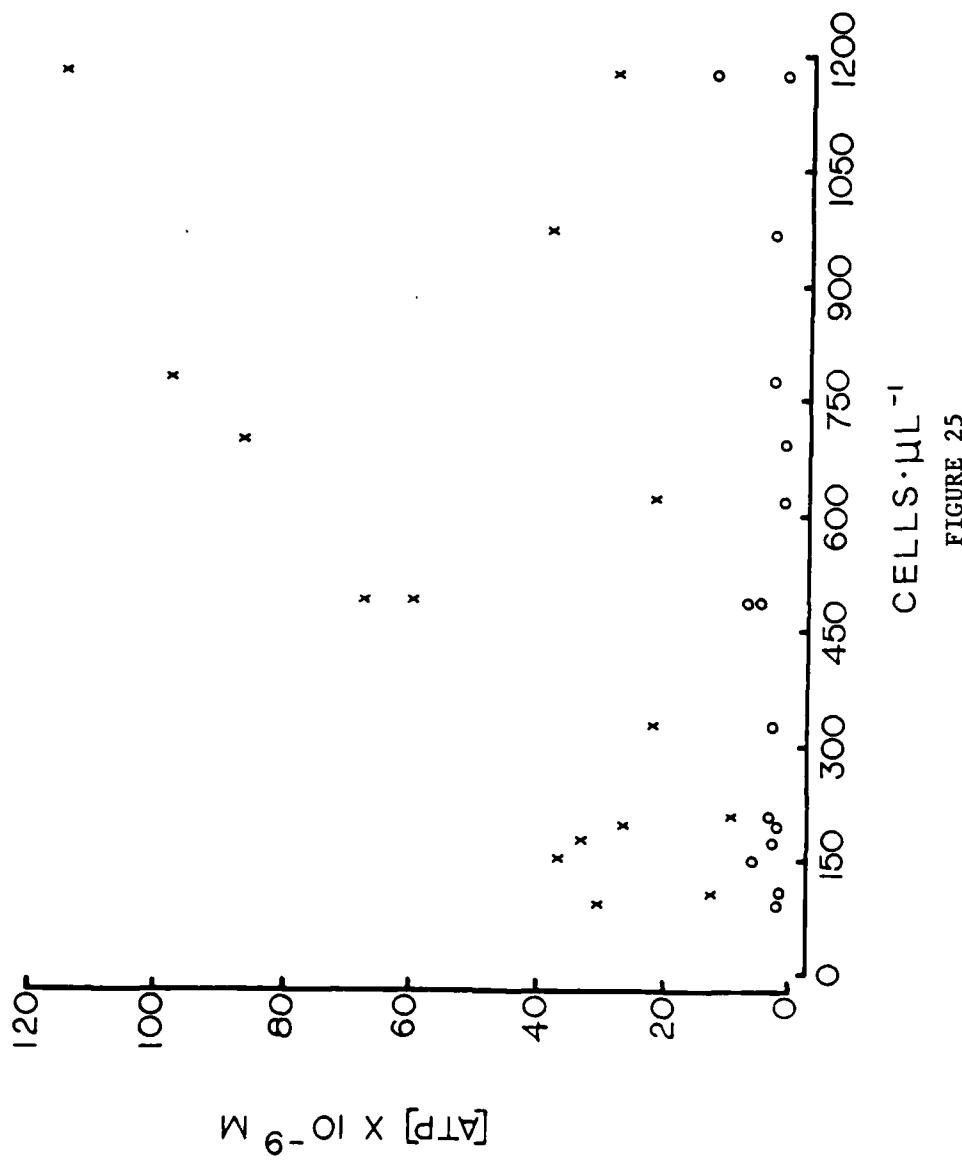
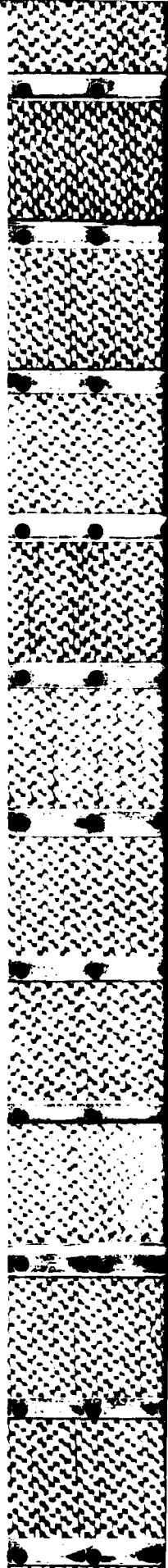


FIGURE 25

FIGURE 26
ATP Concentration in Gently Dispersed Cell Samples
Before (x) and After (Δ) Sonication for 5 Minutes.
Only 60-80% of the cells were lysed following this
procedure. Lysed cells produced significantly higher
ATP levels than dispersed cell samples.



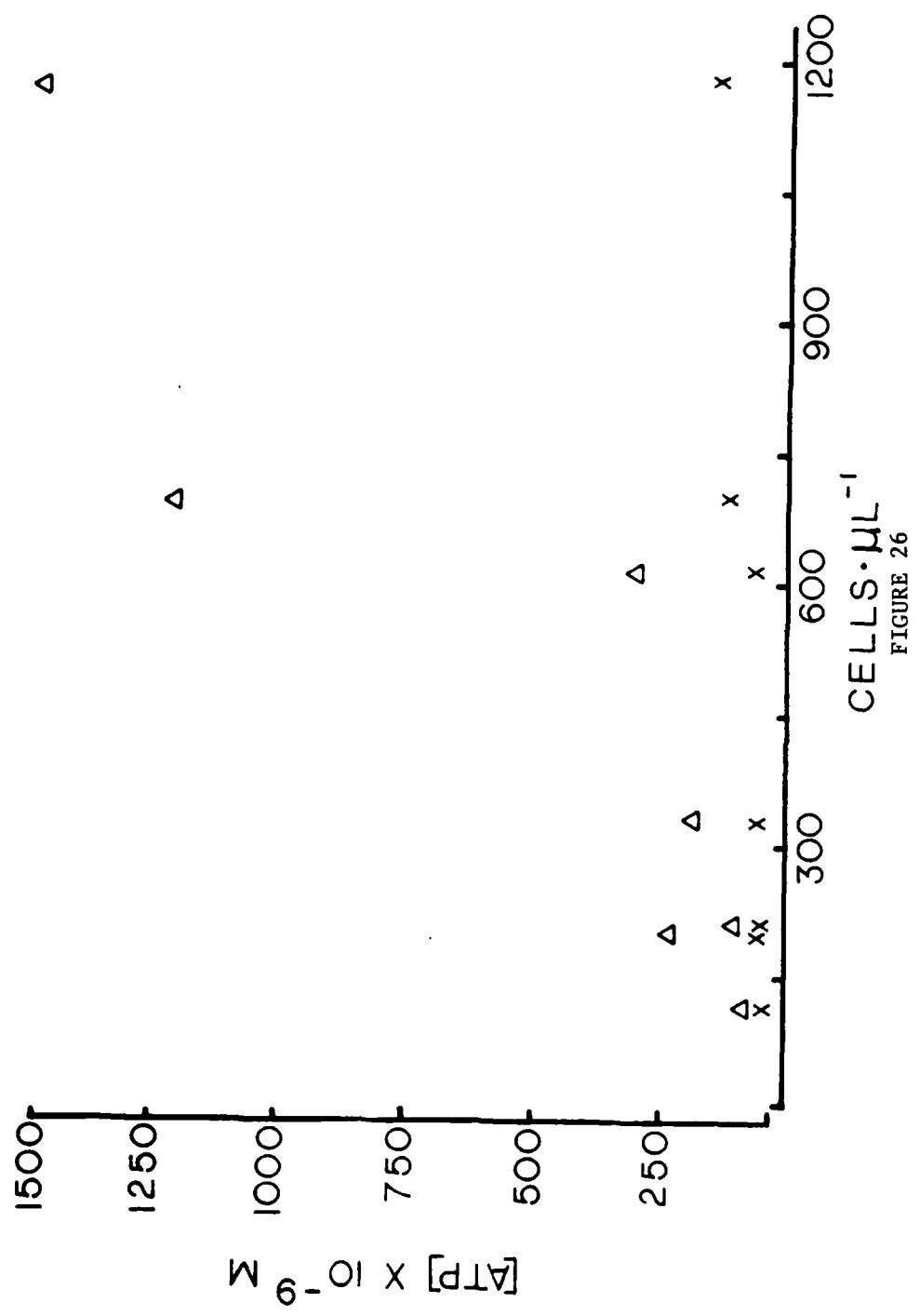


FIGURE 26

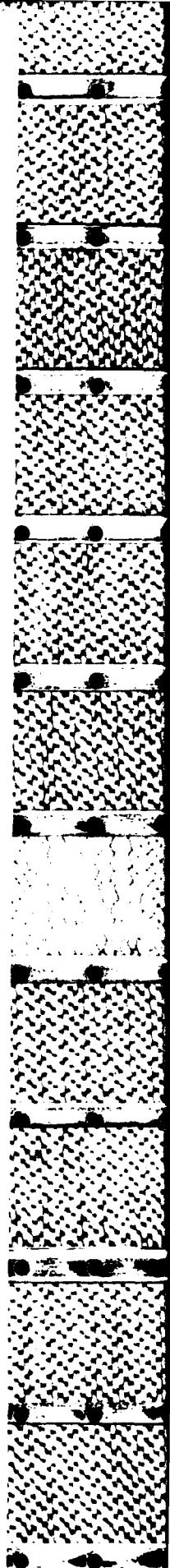


FIGURE 27
ATP Concentration in a Red Cell Sample Allowed
to Settle and in a Sample Continuously Stirred.

Two identical samples of red blood cells (330 cells/ μ l) were assayed three times at hourly intervals. The increasing appearance of ATP in the sample allowed to settle by gravity (o) contrasted with the relatively stable concentration of ATP seen in the sample maintained on a tube rotator (●), which kept the cells constantly moving. Time 0 is when the cells were harvested. The gravity-settled cells were resuspended by gentle mixing prior to assay.

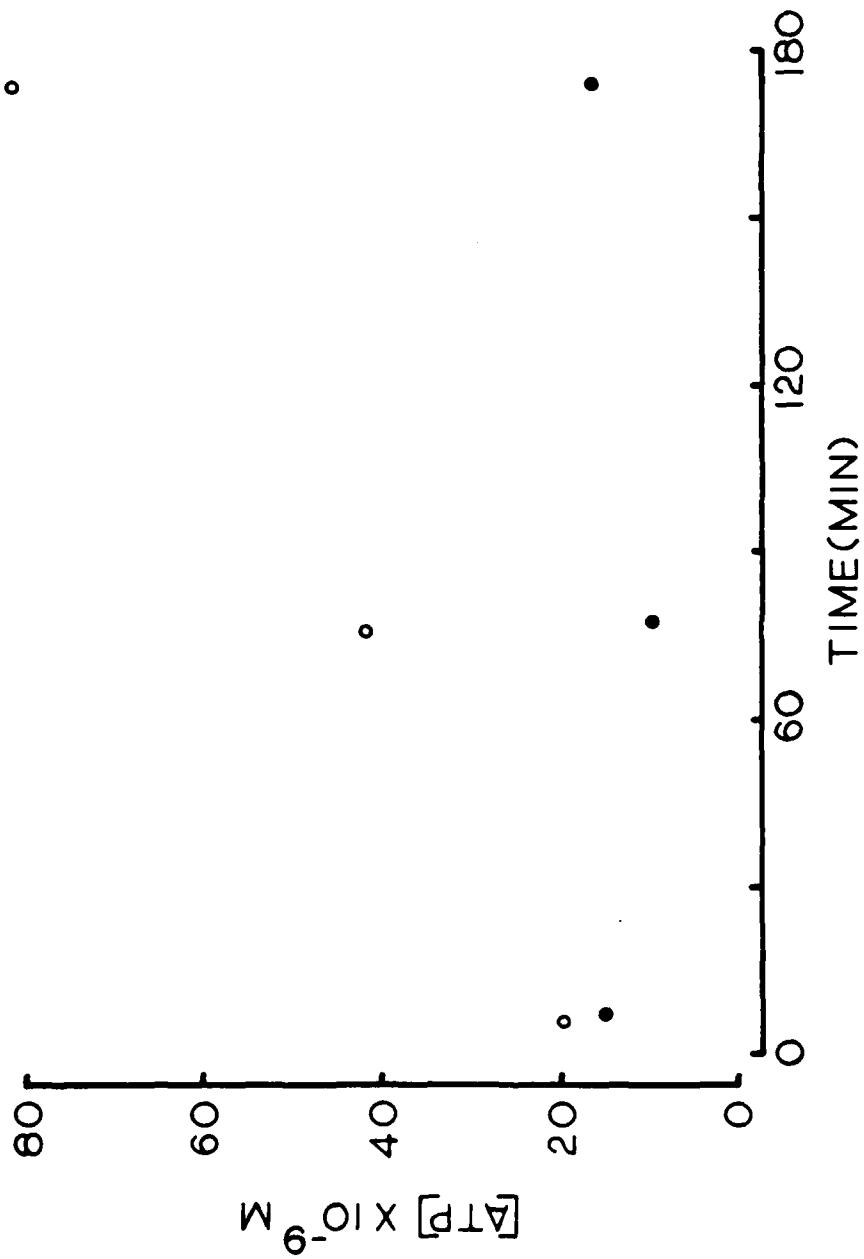


FIGURE 27

On a per-cell basis, the ATP concentration in stirred cells actually had a tendency to decrease slightly. This is shown in Figure 28a. Based on experiments with nine animals, no significant difference was observed between the first and second assay. However, if the cells were allowed to settle by gravity for about one hour, a significant increase in ATP was seen between the first and second assay.

A comparison of the difference between the two assays allowed calculation of ATP release as an efflux (see APPENDIX III for equation) that provided for a statistical comparison between the two experimental procedures. Figure 28b demonstrates that ATP efflux (in molecules/cm²·sec) in settled cells was extraordinary when compared with stirred cells. The latter exhibited almost no ATP efflux.

Ecto-ATPase Activity of Erythrocytes

The reason for the decrease in ATP concentration observed in stirred cells was very likely due to the ATPase activity of the erythrocytes. The ability of frog red cells to hydrolyze ATP has previously been documented at 1.86×10^7 molecules/cell·min (438). Figure 29 shows that the hydrolysis of exogenous ATP added to a cell sample occurred at an exponential rate.

FIGURE 28
Contrast of ATP Associated with Frog
Erythrocytes: Stirred vs. Gravity-Settled.

- a) Cell samples ($n=9$) that were allowed to settle by gravity for approximately one hour released ATP, as measured by the significant increase (*) at an $\alpha = .05$) in the extracellular concentration of ATP compared to the initial assay. When the cells were continuously stirred (see METHODS), there was no significant difference between the first and second assay. The ordinate was calculated from the extracellular assay signal (B in Figure 36) divided by the number of cells assayed.
- b) ATP release quantified as an efflux in molecules/ $\text{cm}^2 \text{ sec}$ (see APPENDIX III) indicates significant release (*) at an $\alpha = .05$) when cells were allowed to settle by gravity. Values indicate mean \pm S.E. in both a) and b).

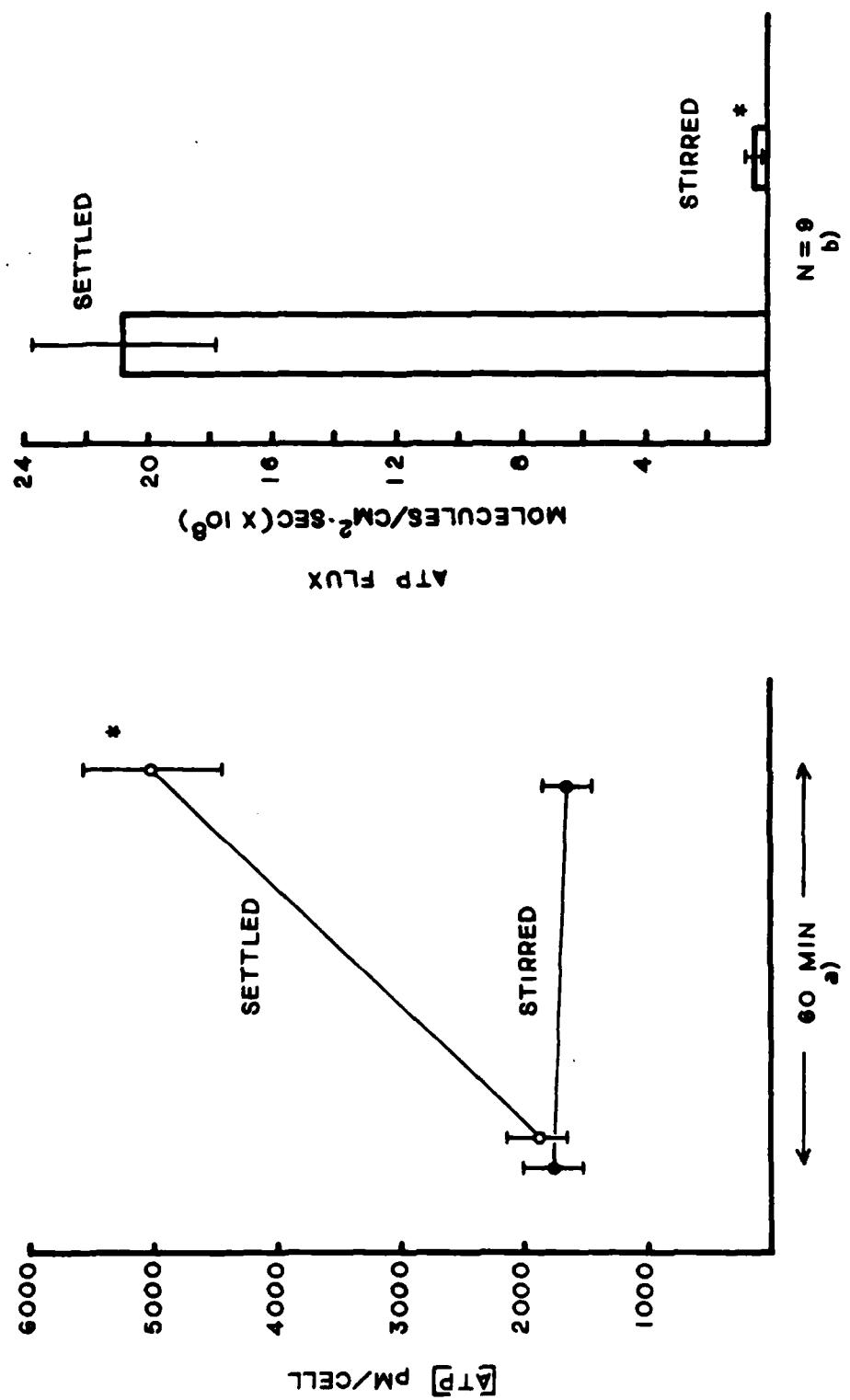


FIGURE 28

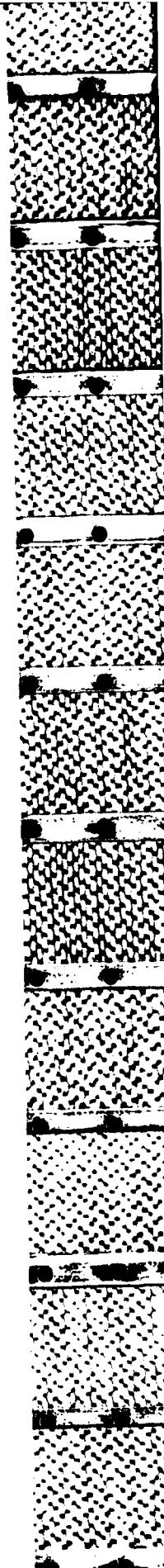
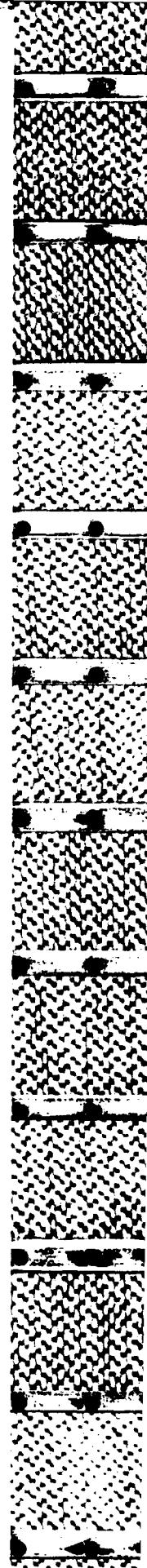


FIGURE 29
Disappearance of ATP Added to Frog Erythrocytes

Exogenous ATP was added to cell samples to make an initial concentration of 500 μ M at time 0. Five successive assays were performed in order to calculate an exponential regression (see Table IV). Samples containing EDTA (Δ) were comparable to those without EDTA (o): there was no inhibition of ATPase activity.



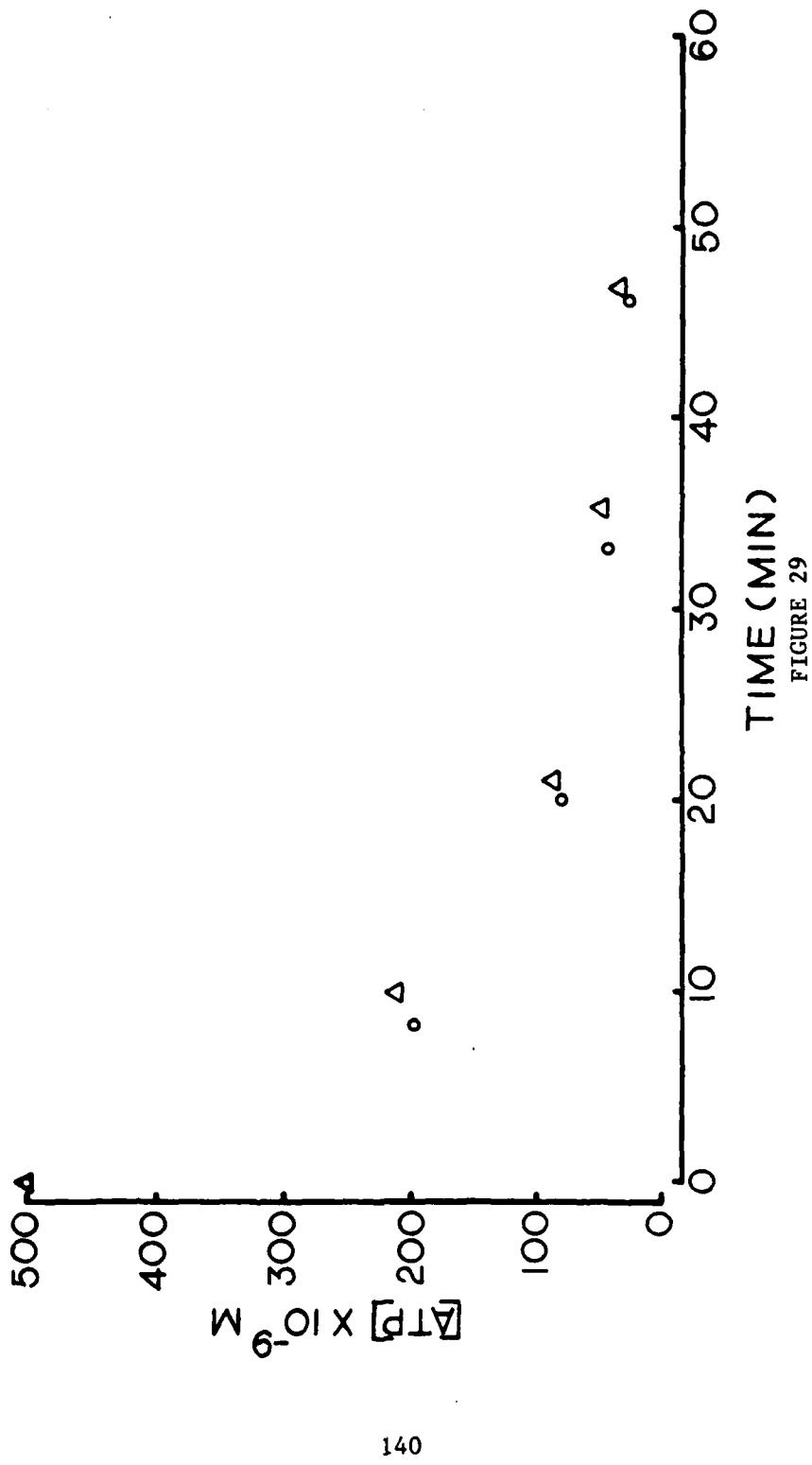
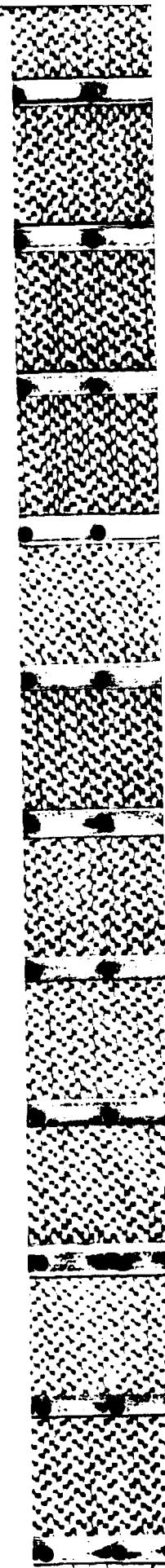


FIGURE 29



Ethylenediamine tetraacetic acid (EDTA), contrary to a previous report (443), did not inhibit this hydrolysis. From an exponential regression of the data, a rate of hydrolysis was calculated (Table IV). The mean rate of hydrolysis for four samples was 2.358×10^7 molecules/cell·min, a number in close agreement with that already reported.

Anion Channel Blockers: ATP Efflux Antagonized

When ATP effluxes from the settled cells, the release mechanism should be susceptible to manipulation. Though nothing was found to enhance the release of ATP without lysing the cells, several substances were effective in inhibiting the efflux of ATP.

One of these substances was probenecid, an inhibitor of cAMP efflux (105). The same protocol as that for settled cells was used. Probenecid (1 mM final concentration) was added to one of two identical cell samples. When compared with control samples having no probenecid, the samples with probenecid exhibited a diminished release of ATP. The second assay after one hour of settling was only slightly increased from the first assay when probenecid was present. This is seen in Figure 30a. Figure 30b shows that a significant decrease in ATP efflux from red blood cells occurred when probenecid was present.

TABLE IV

Rate of ATP Hydrolysis by Frog Erythrocytes

Sample	Rate*	Regression Equation**
1	3.519	$y = 370.8e^{-0.062x}$
2	2.730	$y = 395.2e^{-0.058x}$
3	1.462	$y = 191.4e^{-0.041x}$
4	1.722	$y = 176.5e^{-0.046x}$
mean=	2.358	
s=	0.948	
S.E.=	0.474	

*rate $\times 10^7$ molecules/cell·min**y= ATP concentration $\times 10^{-9}M$

x= time in minutes

An exponential regression was calculated for assay data in which the extracellular ATP concentration was determined at 8-12 minute intervals following the addition of ATP (Samples 1 & 2, 500 nM concentration; Samples 3 & 4, 250 nM concentration). The regression estimate for the ATP concentration at minute 4 or 5 following addition of ATP was used to calculate the rate of hydrolysis according to the equation provided in Appendix II.

FIGURE 30
Inhibition of ATP Efflux from Nucleated
Erythrocytes by Probenecid (PRO).

- a) Allowing cells to settle by gravity (control) for approximately 60 minutes caused the extracellular concentration of ATP to increase significantly (* at an $\alpha = .05$) over the initial value. The difference between the first and second concentration was not significant when probenecid (1 mM) was present, indicating decreased release of ATP from the cells. The ordinate was calculated from the extracellular assay signal (B in Figure 36) divided by the number of cells assayed.
- b) ATP release calculated as an efflux in molecules/cm²·sec (see APPENDIX III) indicates a significant inhibition (*) at an $\alpha = .05$) when probenecid was present. Probenecid (1 mM), an anion channel blocker, caused almost a 10-fold decrease in the efflux of ATP. Values indicate mean \pm S.E. In both a) and b).

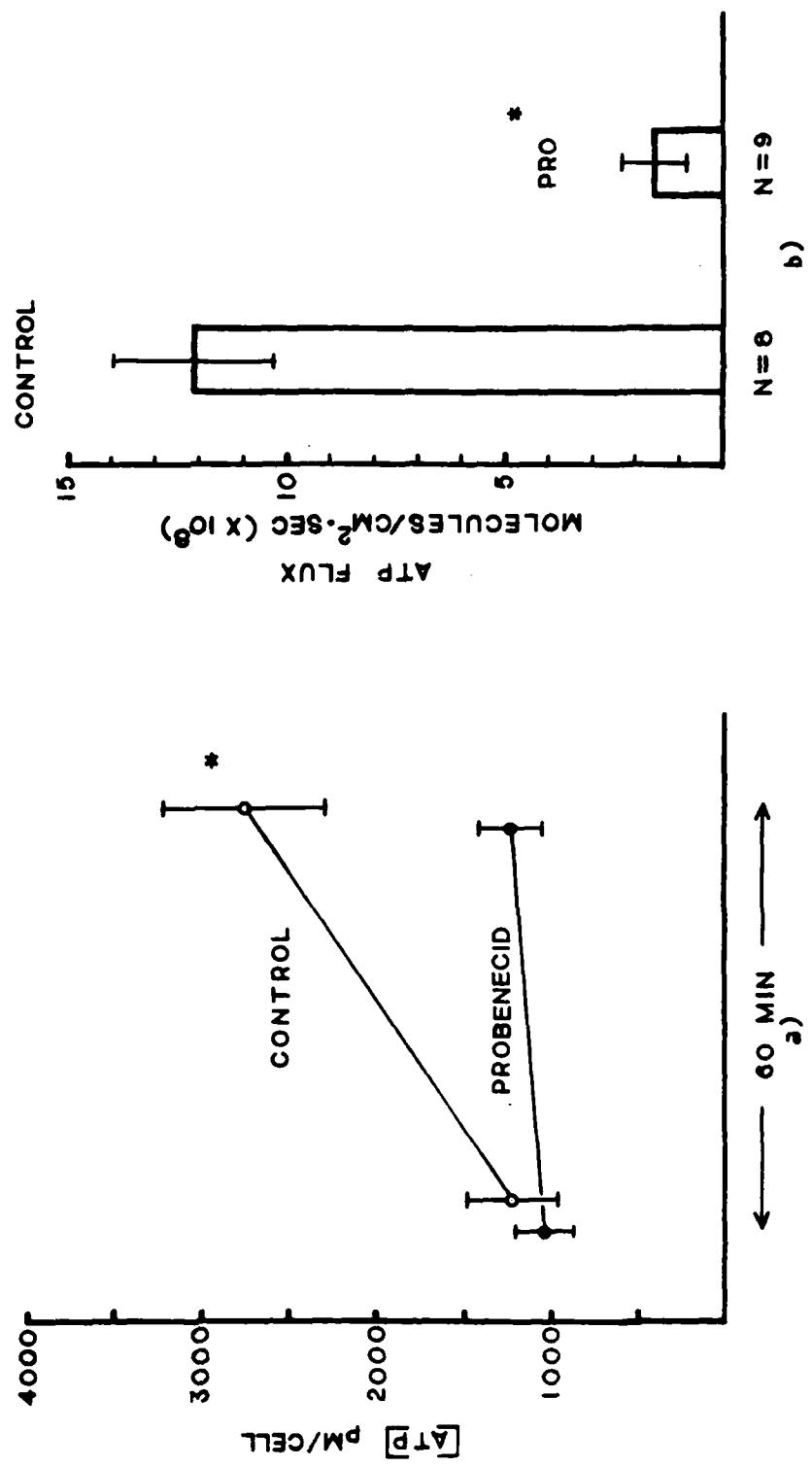
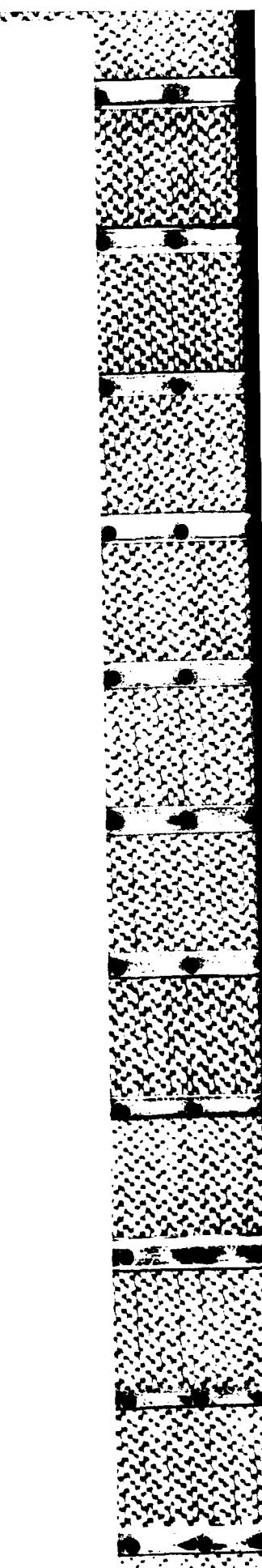


FIGURE 30



A similar display of inhibition was possible with furosemide, an anion channel blocker (142). At a final concentration of 1 mM, it was able to decrease the appearance of ATP (Figure 31a). In terms of efflux, furosemide was able to significantly diminish ATP release from these cells. By comparison with probenecid, furosemide appeared to be less potent, providing only a four-fold inhibition as compared to 10-fold with probenecid (Figure 31b).

Further evidence that this phenomenon was an efflux inhibition was based upon evidence using atractyloside. At a concentration of 1 mM, atractyloside, an ADP-ATP translocator protein inhibitor, was used to decrease the concentration gradient of ATP from the inside to the outside. Using the same protocol as described before, a considerable decrease in ATP efflux was observed when cells were allowed to incubate for one hour in atractyloside. The period of time was probably not adequate to produce a maximum effect on ATP efflux, since the difference in flux between control conditions and with atractyloside present was not statistically significant (Figure 32).

Confirmation of Extracellular ATP

The appearance of ATP near the settled cells at a concentration greater than observed at the surface of the supernatant fluid above the cells raised the first of several questions concerning cell lysis. Were the cells spontaneously lysing in the frog Ringer's solution?

FIGURE 31
Inhibition of ATP Efflux from Nucleated
Erythrocytes by Furosemide (FUR).

- a) Same control conditions as in Figure 30 (* significant at an $\alpha = .05$). With furosemide (1 mM) present, ATP concentration in the second assay 60 minutes later was not statistically different, indicating decreased release of ATP from the cells under these experimental conditions. The ordinate was calculated as in Figure 30.
- b) Furosemide (1 mM), an anion channel blocker, caused an approximate 4-fold inhibition (*) significant at an $\alpha = .05$) in ATP efflux (see APPENDIX III for efflux calculation). Values indicate mean \pm S.E. in both a) and b).

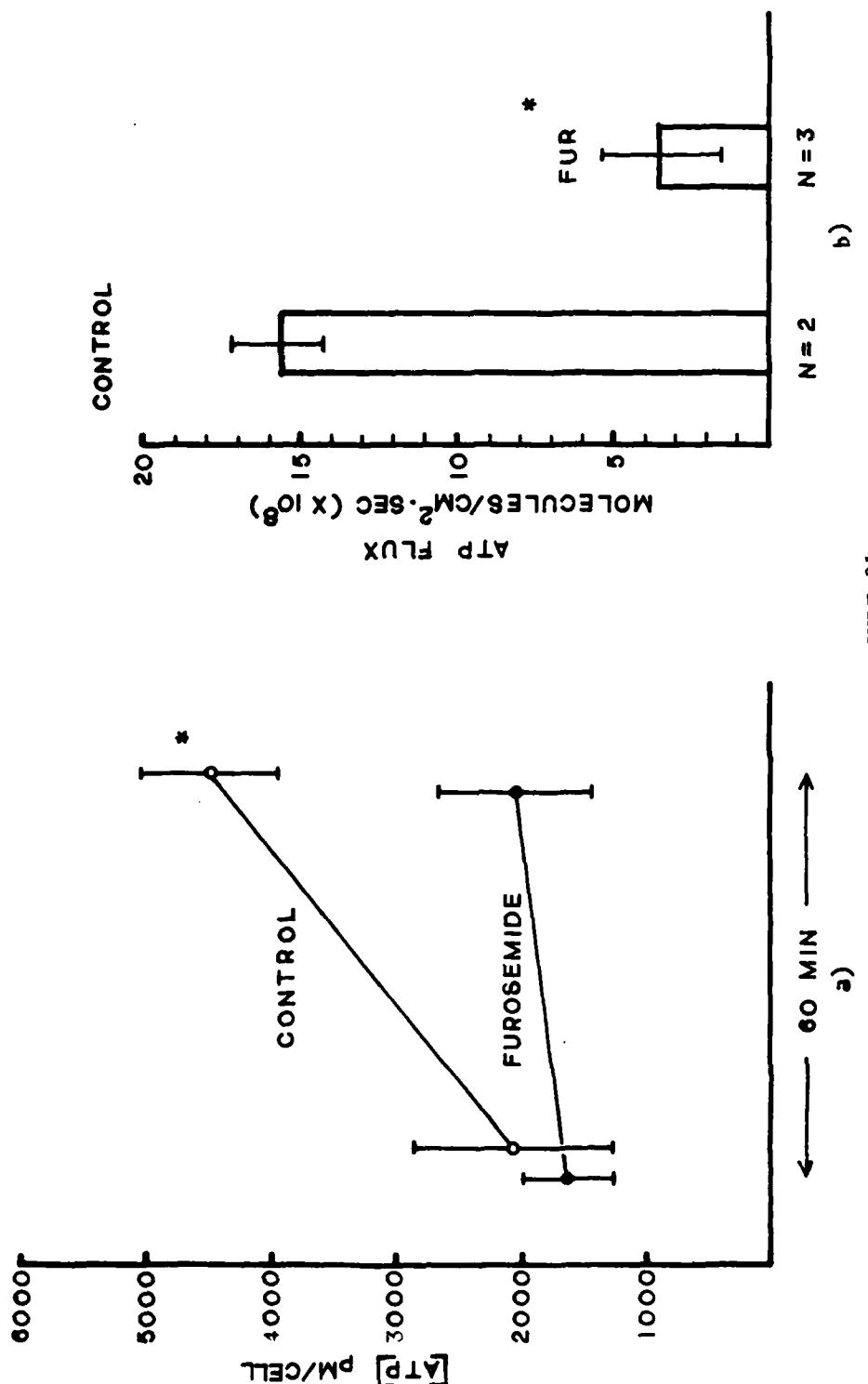


FIGURE 31

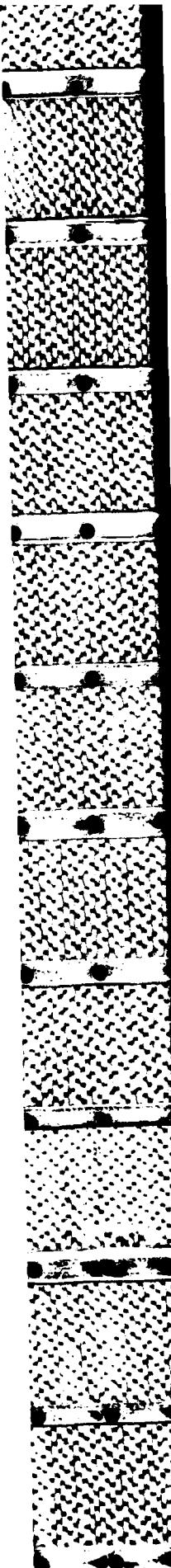


FIGURE 32
Atractyloside Effect on ATP Efflux
from Nucleated Erythrocytes.

Control conditions were the same as in Figure 30. There was no statistical difference between ATP efflux during control conditions and when atractyloside (1 mM) was present.

Atractyloside, an inhibitor of the ADP-ATP translocator protein of the inner mitochondrial membrane (254,468), was used to decrease intracellular ATP concentration of the cells. This resulted in a tendency for ATP efflux to decrease from these cells, an observation possibly of statistical significance if examined following incubation with atractyloside over a longer period of time.

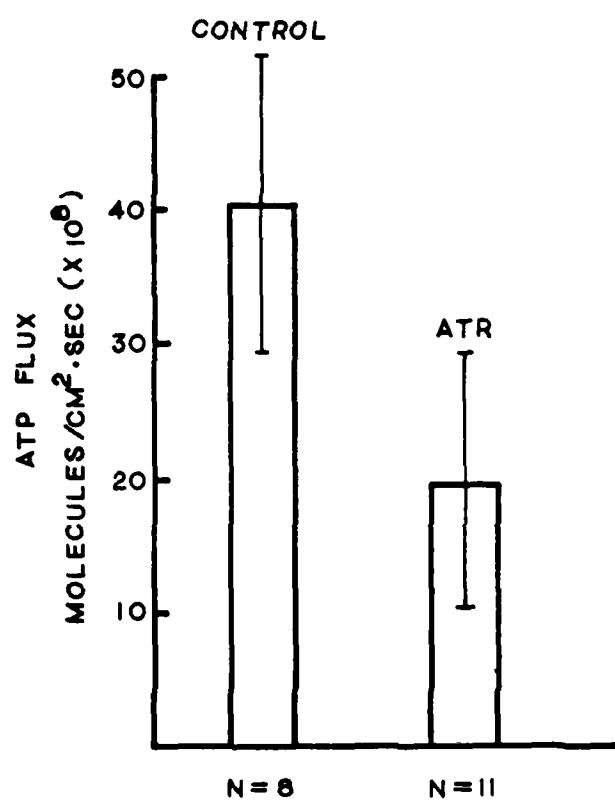


FIGURE 32

During the cell counting procedure, not one cell was observed to lyse. Counts were made before and after 60 minutes of settling without a statistically verifiable decrease from one count to the next. For example, five identically prepared cells samples varied slightly in the number of cells per microliter, but the mean and standard deviation of the number of cells per sample before settling were virtually the same following settling.

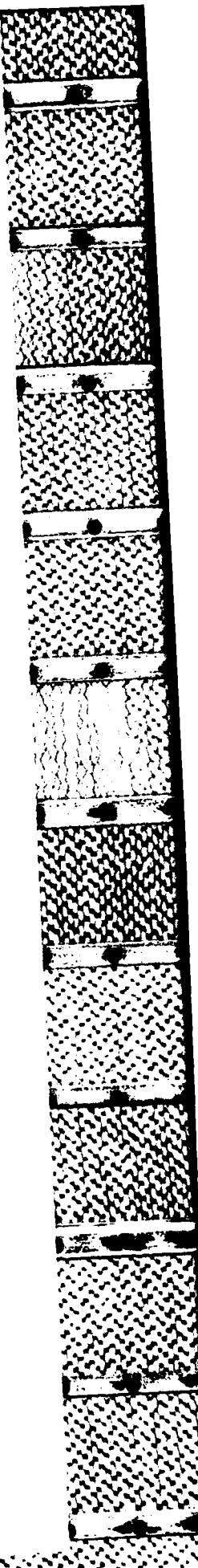
Figure 33 provides evidence that spontaneous lysing was unlikely. In a cell sample in which the count was repeated 25 times during a 90 minute period, the counts near the end exhibited the same variability about the mean as did the early counts. Were the cells spontaneously lysing in the frog Ringer's solution, the number of cells would have decreased over time.

Cell lysis also could have been occurring during aspiration of the cells into or out of the 20 gauge needle during the assay procedure. Frog cells are greater than 20 microns in diameter. It seemed possible that some cells may have lysed during this procedure.

If lysis were occurring in this manner, some variability from one assay to the next would have been noticeable in the stirred samples. This was not observed. Indeed, lysis was not observed using deliberate repetitive aspirations of the same sample. This is shown in Table V. Seven aspirations of four different cell samples did not decrease the number of cells as the number counted in the two successive counts were within a 10% standard deviation.

FIGURE 33
Iterative Count of a Cell Sample.

Variability occurs in hemocytometer counting, as shown by the 25 individual counts (o) made over a 90 minute period. Significantly, the count varied around the mean (solid line) and seldom digressed more than one standard deviation (dashed line) away. This indicates the cells were not spontaneously lysing in the frog Ringer's solution.



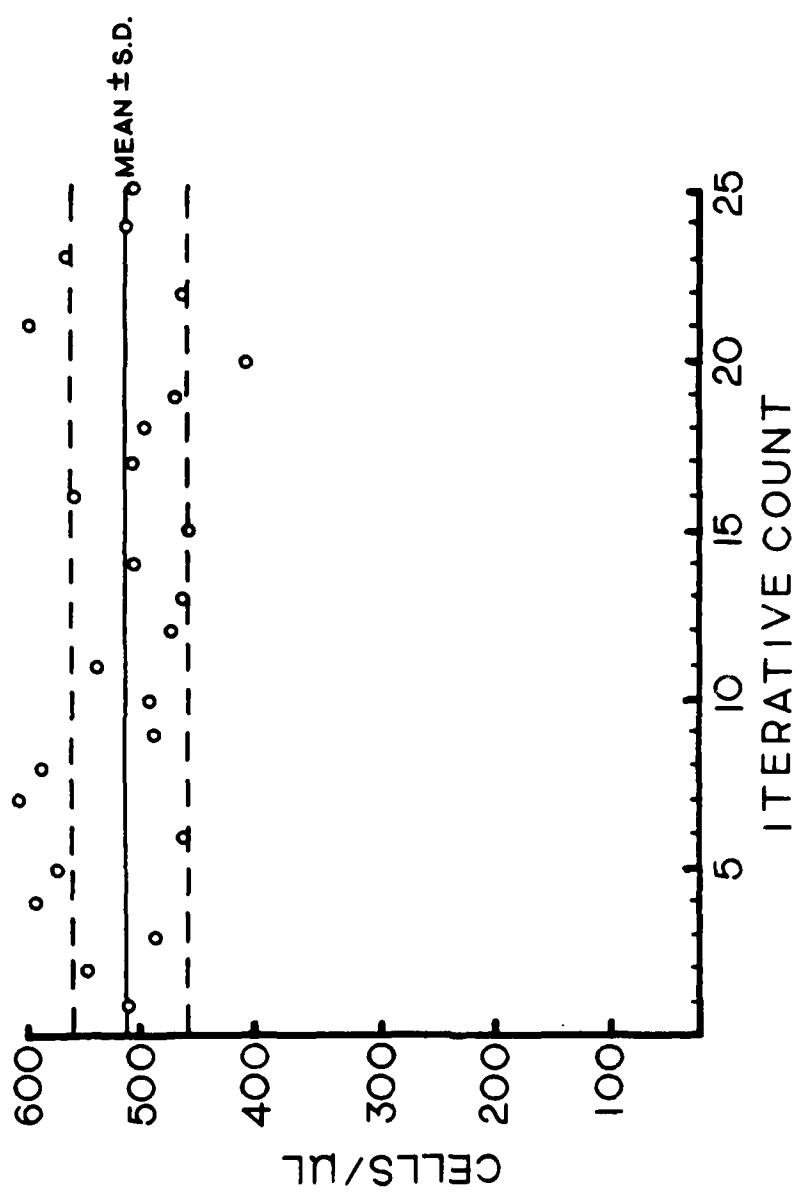


FIGURE 33

TABLE V
Effect of Aspiration on Cell Counts

Sample	1st Count	2nd Count
1	340	320
2	590	575
3	805	900
4	1275	1150

Cell samples of 1.4 ml volume containing different numbers of cells were counted by hemocytometer, then transferred to another plastic microvial by micropipetting 200 μ l at a time aspirating through a 20 gauge needle, and then re-counted. Seven aspirations per sample were required, a process that appeared to have caused little, if any, lysis.

It is unlikely that this procedure resulted in lysis and contributed to an increase in the amount of ATP because a single assay involved movement of the frog red cells only once up and down the 20 gauge needle. This statement about frog cells can be made with conviction because it was not found to be applicable to salamander cells.

Red cells of Amphiuma tridactylum are the largest known erythrocytes, measuring over 70 microns in diameter. Extracellular ATP also was discovered to be associated with these nucleated red cells. Yet, an effort to study this ATP was curtailed because of the size of the cells and the limitations of the assay apparatus. These cells lysed when the same procedures for frog red cells were used. This lysis was verified by the extreme variability in the ATP concentration of samples from one assay to the next.

Another question was the following. Were the frog red cells lysing upon contact with the firefly assay solution? A considerable degree of variability would be expected if this occurred. Yet, successive assays within minutes of each other generated virtually identical recordings of the light signal generated by a sample, indicating that lysis was not probable.

A parallel issue to this question was the similarity of the recorded light signals generated by cell suspensions and standard solutions of ATP. When compared, exceptional similarity was observed. Cell suspensions that generated recordings in millivolts could be superimposed on the recordings generated by ATP standards. Figure 34 demonstrates that the recordings of the light signals were practically identical in terms of the rate of rise of the light emitted and the exponential decay. It is concluded that the ATP assayed in the cell suspensions had to have been already present to account for such similarity between recordings. If the cells were lysing and releasing their intracellular ATP while in the assay solution, the rate of rise would have been slower and the signal decay would not have been exponential.

The development of one experimental procedure allowed verification of this. It was reasoned that, if the cells had not lysed, they would still be present in the firefly assay solution in front of the photomultiplier tube (PMT). Then, exposure of the cells to a detergent that did not affect the firefly bioassay but lysed the cells would release the intracellular ATP. The detergent that was used was digitonin.

FIGURE 34
Superimposed Recordings of Light Emissions by
(S) Standard Solutions of ATP and (C) Cell Suspensions.

Note the similarity of the time course of the exponential decay and the rate of development of the light signal in both the standards and cell samples, indicating the presence of ATP in the fluid surrounding the cells. i- 10 nM ATP and 85 cells/ μ l; ii- 25 nM ATP and 275 cells/ μ l; iii- 100 nM ATP and 2225 cells/ μ l; iv- 250 nM ATP and 1850 cells/ μ l. Vertical bar, 10 mv in "i" and "ii", 100 mv in "iii" and "iv". Horizontal bar, 15 seconds in each.

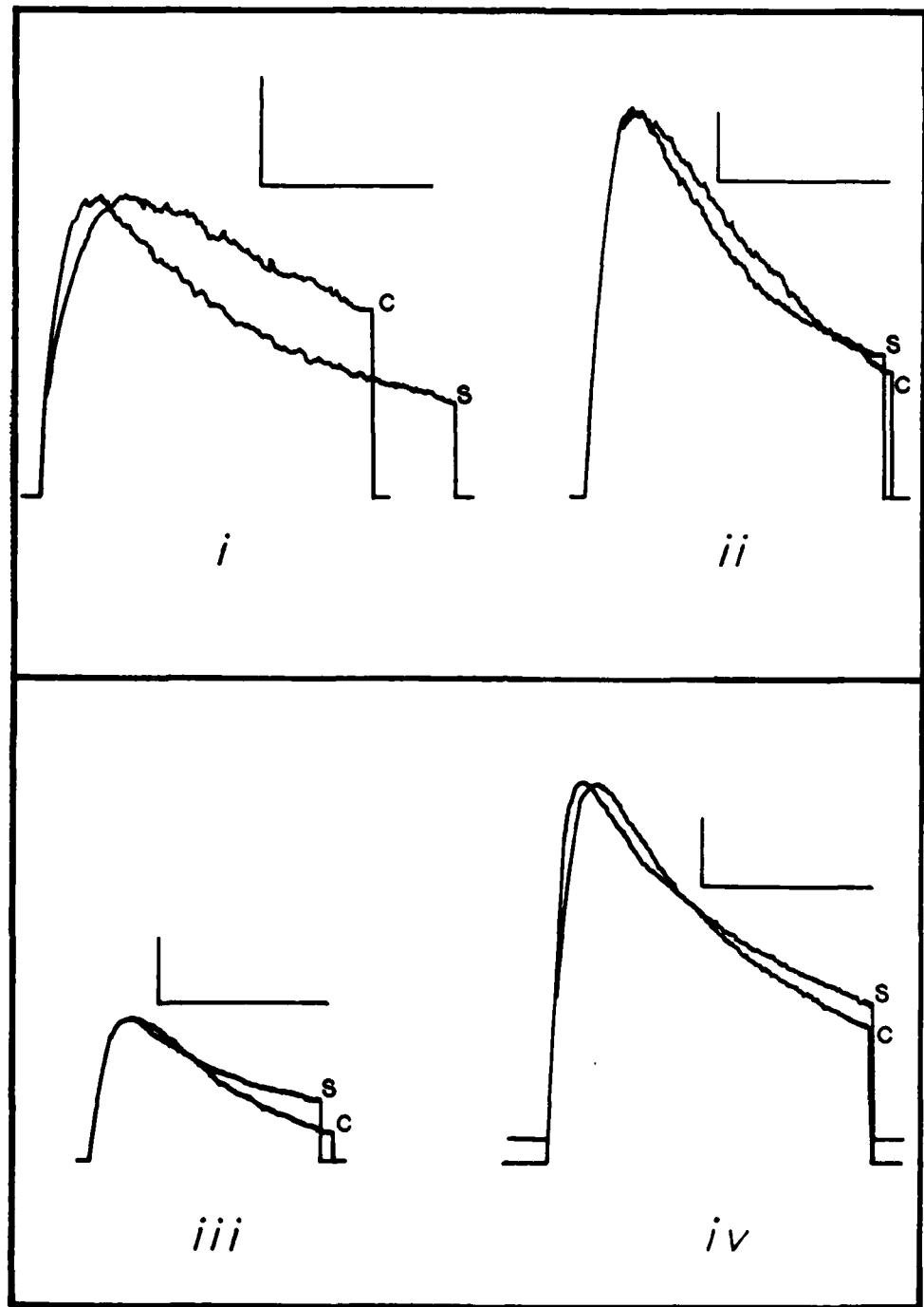


FIGURE 34

Figure 35 shows that at least a 5 μ M concentration of digitonin was required to lyse frog red cells. This was based upon cell counts that resulted in finding nothing but cell debris upon microscopic examination.

It was also possible to watch the cells lyse when digitonin was added to a cell sample under the cover slip. Almost instantaneously as digitonin reached the cells, the cell membrane disintegrated, spilling the cytoplasmic contents and collapsing some of the lipid remnants around the nucleus. The nucleus was observed to decrease in size, but usually did not disintegrate completely.

The application of digitonin to the cells in front of the PMT also resulted in their lysis, proving that they were still present in the firefly assay solution. This procedure is completely described in Figure 36. It must be emphasized that signal B in this figure is the assay signal on which experimental comparisons were made. Significantly, signal B is the measure of extracellular ATP associated with the frog red blood cells.

Allowing the cells to settle caused an increase in the ATP efflux from the cells, as measured by an increase in this signal. Stirring the cells or having probenecid, furosemide, or atracyloside present inhibited ATP efflux, as seen by little or no change in this signal from one assay to the next. It can be

FIGURE 35

Concentration of Digitonin Required to Lyse Frog Erythrocytes.

Various concentrations of digitonin were added to cell samples that had already been counted. Counting a second time allowed the determination of the percent of lysis by digitonin. Note that at less than 5 μ M digitonin final concentration, cell lysis was incomplete, whereas all cells were lysed at concentrations greater than 5 μ M.

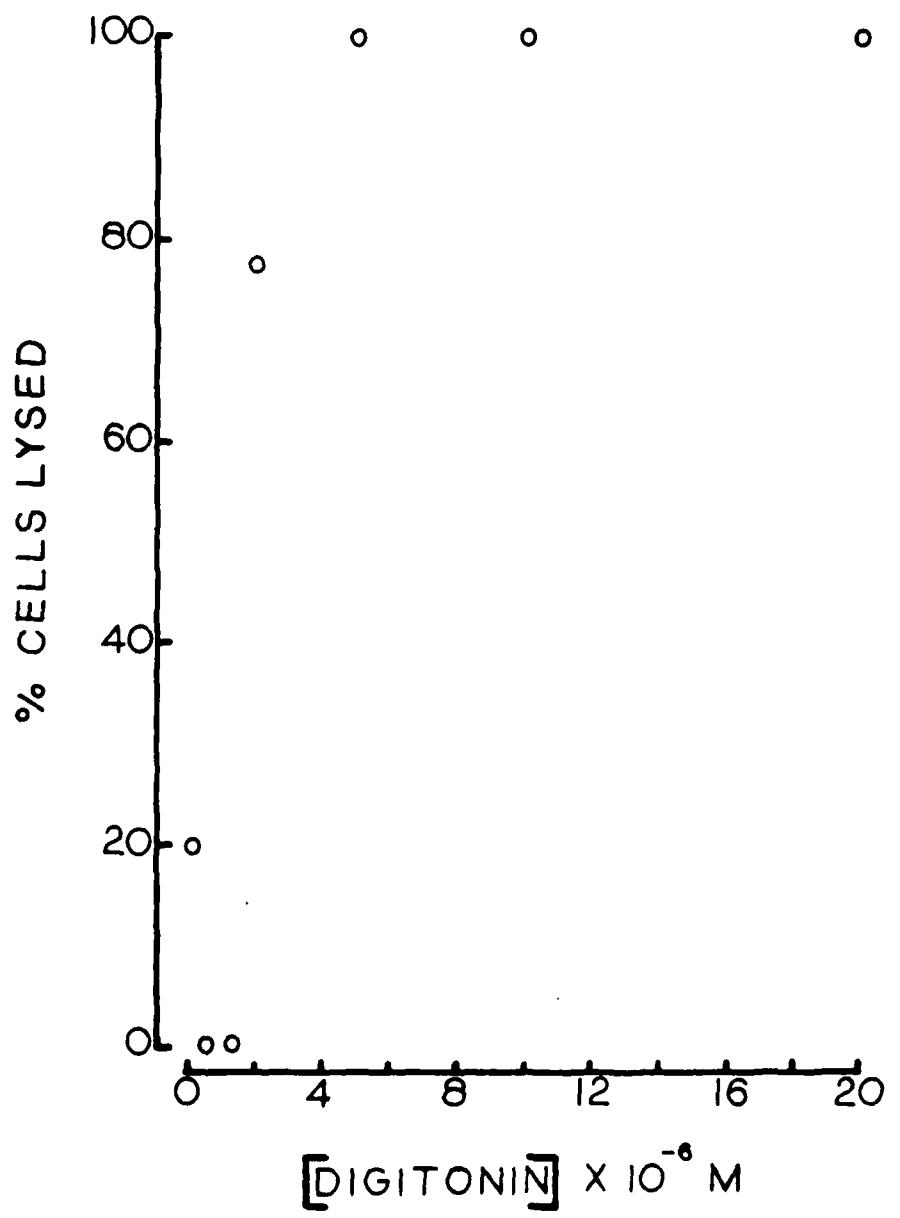


FIGURE 35

FIGURE 36
Recording of the Light Signal Generated
by an Assay of a Cell Suspension.

This recording demonstrates the real-time hydrolysis of ATP.
A- the background signal produced by the firefly reaction mixture (reconstituted firefly lantern + synthetic luciferin) alone. This is followed by a diminution in the light signal upon addition of the cell sample to the firefly bioassay mixture.
B- assay signal produced by the hydrolysis of the extracellular ATP associated with nucleated red cells. Note the rapid-rise increase in the light signal. This is followed by the exponential decay in the signal indicating that most of the ATP has been hydrolyzed. At the arrow, 200 μ l of 5 μ M digitonin was added to the cuvette. Ten to twelve seconds later signal C resulted.
C- assay signal produced as digitonin lysed successively more and more cells, releasing their intracellular content of ATP. Note the slow-rise increase in the light signal, and lack of exponential decay because cells were still lysing. This experimental procedure demonstrated that extracellular ATP was being measured (Signal B) and that the cells were not lysing while in the firefly bioassay solution.

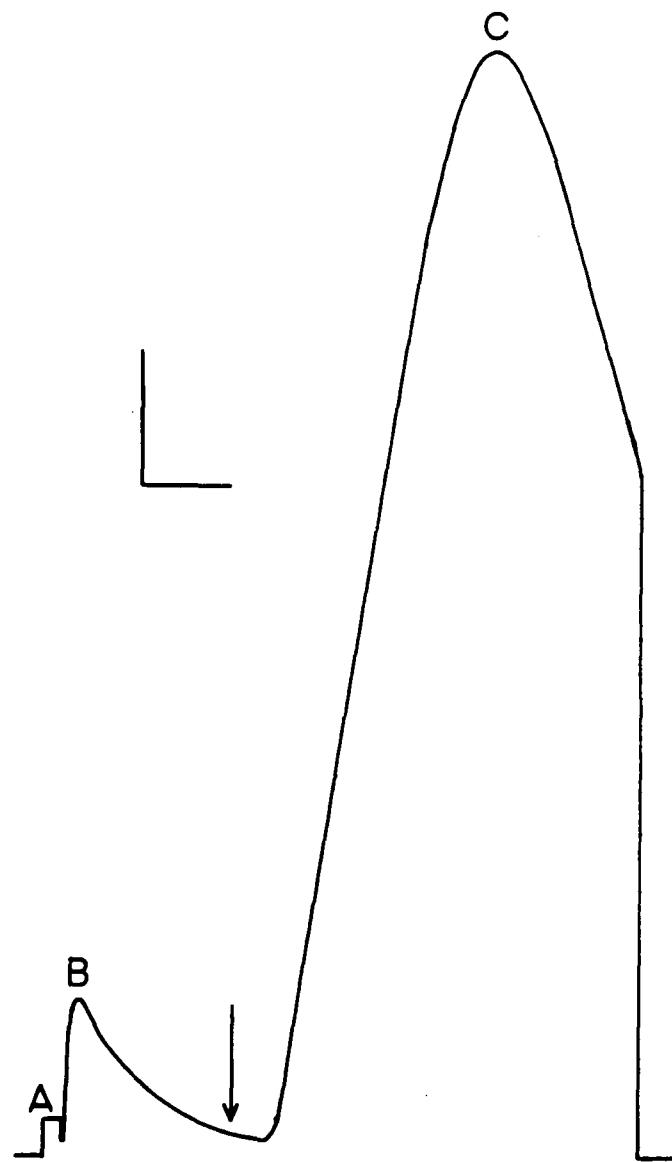


FIGURE 36

concluded that extracellular ATP was being measured and that calculation of an efflux was justified. Based on the evidence presented in this section, it can also be concluded that the cells were not lysing either spontaneously, during aspiration at assay, or upon contact with the firefly bioassay solution.

A final piece of evidence confirms that ATP was the substance that was associated with the erythrocytes. Figure 37 demonstrates that apyrase, an enzyme that hydrolyzes ATP to AMP, abolished the light signal generated when a cell sample was assayed. Thus, it is logical to conclude that ATP was responsible for the light signals recorded when cell samples were assayed.

Intracellular ATP of Frog Erythrocytes

The intracellular ATP concentration ($[ATP]_i$) of frog erythrocytes has been determined by chromatography (24) and high performance liquid chromatography (52) as 6.10 mM and 3.31 mM, respectively. Using the firefly bioassay, $[ATP]_i$ was determined using three different procedures. Multiple cell samples from a frog were sonicated or lysed with digitonin, and then assayed for $[ATP]_i$, or the cells were placed in the firefly assay solution in front of the PMT and then lysed with digitonin. An average $[ATP]_i$ was determined for each method. The equation provided in APPENDIX I was used to calculate the $[ATP]_i$. It makes the

FIGURE 37

Light Signal Generated by Extracellular ATP in the Medium Around Erythrocytes, and Following the Addition of Apyrase.

i- light signal generated by the extracellular ATP of frog red cells;
ii- light signal generated after addition of 1.05 mg of apyrase. The apyrase hydrolysed all of the ATP and abolished the assay signal. Vertical bar, 100 mv in "i", 10 mv in "ii". Horizontal bar, 20 seconds in each.

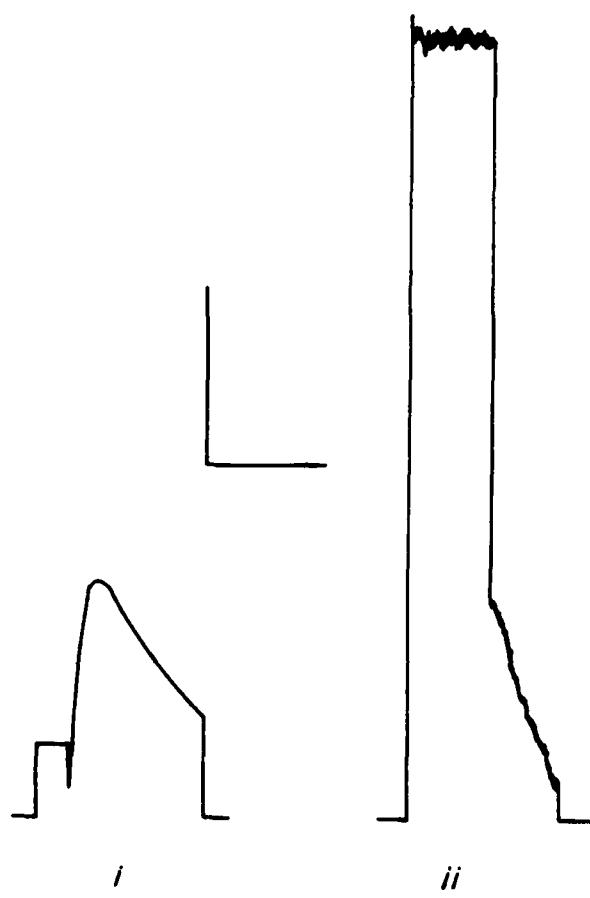


FIGURE 37

assumption that the mean cell volume was $800 \mu^3$ (193), that 65% of the red cell was water (114) and that the nuclear volume vis-a-vis water and ATP was no different from the cytoplasm (103).

Table VI summarizes the findings with seven different animals. Both the sonication and digitonin procedures provided similar $[ATP]_i$ values. The overall mean \pm S.E. for twenty-nine observations on six animals using the first two procedures was 2.9 ± 0.3 mM. The third procedure of lysing the cells in front of the PMT provided ATP levels that averaged less than 1 mM (see p. 196).

Intracellular ATP of Salamander Erythrocytes

No reference was found for the intracellular concentration of ATP in Amphiuma tridactylum. Using the firefly bioassay procedure, $[ATP]_i$ was determined by lysing the cells with digitonin ($20 \mu\text{M}$). Cell samples containing different numbers of cells were counted, lysed, and then assayed. An average $[ATP]_i$ was determined for each animal based on multiple observations. Again, the same equation as used for frog red cells (see APPENDIX I) was used for this calculation. It assumed a mean cell volume of $15,000 \mu^3$ (262), that 65% of the red cell was water (114), and that water and ATP in the nuclear volume was the same as in the cytoplasm (103). Table VII provides the mean $[ATP]_i$ for two animals. The overall mean \pm S.E. for twelve observations was 1.7 ± 0.3 mM.

TABLE VI
 $[ATP]_i$ of Frog Erythrocytes

Animal	Observations	[mM] mean \pm S.E.
SONICATION		
1	3	4.6 \pm 0.5
2	5	1.6 \pm 0.1
3	6	2.0 \pm 0.8
DIGITONIN		
1	9	3.4 \pm 0.3
2	3	3.6 \pm 0.7
3	3	2.7 \pm 1.0
DIGITONIN IN FRONT OF THE PMT		
1	3	0.5 \pm 0.03

Multiple observations using different cell counts were made for each animal, and the average $[ATP]_i$ determined. The sonication procedure lysed no more than about 80% of the cells, so an appropriate adjustment in the $[ATP]_i$ was made. Digitonin at a concentration of 20 μ M was used in the second procedure. It was added to the whole cell sample, and then 200 μ l of the lysed sample was assayed. In the third procedure, 200 μ l of the cell sample was placed in the firefly mixture in front of the photomultiplier tube (PMT). To this was added 200 μ l of digitonin to lyse the cells. Appropriate volume-dilution adjustments were made in each procedure. For example, adding 200 μ l of digitonin diluted the assay volume by one third. The $[ATP]_i$ was then adjusted upward by one third. See APPENDIX I for calculation method.

TABLE VII
[ATP]_i of Salamander Erythrocytes

Animal	Observations	[mM] mean \pm S.E.
1	6	1.2 \pm 0.1
2	6	2.2 \pm 0.4

Multiple observations using different cell counts were made for each of two salamanders. Cells were counted in each sample and then lysed by adding digitonin at a concentration of $20\mu M$ to the whole sample. Afterward, $200\mu l$ of the lysed sample was assayed. The same calculation as for frog erythrocytes was used (see APPENDIX I) except that a mean cell volume of $15,000\mu^3$ was substituted in the equation.

DISCUSSION PART I: METHODS

Assay Procedure and Technique

Photinus pyralis (firefly) hydrolyses ATP in order to produce light via luciferyl adenylate. A sensitive bioassay can be produced from reconstituted firefly tails (firefly lantern extract, FLE), allowing the determination of ATP concentrations in the range of 10-2500 nM using the equipment shown in Figure 1. The technique that was employed has been a satisfactory technique for almost two decades. It was recently improved by the addition of synthetic luciferin (237), which increased the sensitivity and allowed differentiation of unknown samples at levels less than 10 nM.

The assay procedure utilizes a photomultiplier tube (PMT), which necessitates that experiments be conducted in absolute darkness or that the FLE mixture (FLE + luciferin) and PMT be isolated in a light-tight box. The latter procedure, similar to that described by Silinski (394) and utilizing an injection port capable of accepting a 20 gauge needle, was used in order to record the complete light signal produced by the firefly during each assay. The peak of this light signal was essential to the accurate measurement of the ATP concentration, since it is the peak signal that is proportional to the amount of ATP present. Also, examination of the fast rise in the signal and its exponential decay allows verification that ATP, rather than some other nucleotide, is the substance being measured.

It is surprising that some researchers are still performing firefly assay procedures in a way that precludes accurate measurement of the ATP concentration. For example, some have measured the light emission after a delayed period following the mixing of the FLE and an unknown sample. Wulf et al. (474) never observed accurate readings because, among other inadequacies, light emission was measured a number of seconds after the peak of the light signal. This measurement was at a time when the recording was on the downslope of Component B in Figure 3. Addanki et al. (3) and Aledort et al. (10) also took measurements on the extreme downslope of the light signal. This was done out of necessity because a liquid scintillation counter (LSC) was used. The LSC procedure, in effect, integrates the area under the curve because it counts photons emitted during the enzymatic reaction. Unfortunately, LSC precludes an accurate count of the total light signal because the counter is not switched on when the FLE and unknown sample are combined. None of these authors described any methodology for dealing with this problem. Neither did they describe methods for eliminating error due to background counts or due to variable luciferase activity from one assay to the next. Methods were employed in the present protocol to eliminate such errors.

Crude FLE has some ATP in it, necessitating that some method be designed to exclude its effect on the measurement of the unknown sample. This was accomplished in two ways, first by waiting 30 minutes for the ATP intrinsic to the FLE to be hydrolyzed before beginning the assay procedure. Second, a signal-to-background (B/A) ratio was mathematically calculated for all standards and unknowns, and from this value was subtracted the signal-to-background ratio for a blank. The signal generated by assaying a blank, which contained no exogenous ATP, was a measure of the previously existing ATP in the FLE. By subtraction of its B/A ratio, the error introduced by the ATP in the FLE was eliminated.

Another error, that due to changes in enzyme activity, was also eliminated by calculating the B/A ratio. Each assay cuvette contained 100 μ l of FLE and 100 μ l of synthetic luciferin. Yet, the enzymatic activity of these two reactants combined varied depending on the accuracy of pipetting and the amount of luciferase enzyme or luciferin actually transferred to the cuvette. The variance was small, but nevertheless could be measured. Consequently, the number of photons produced by the PMT on a real-time basis caused the recorded signal to vary slightly even with a standard solution of ATP. The true measure of the ATP present, then, was not related just to the peak of the assay signal (Component B of Figure 3). It was also dependent on the

concentration of reactants to which the ATP was added, reflected by the height of the background signal (Component A of Figure 3). Thus, the true measure of the ATP was related to the ratio of the assay signal to the background signal (B/A ratio).

The enzymatic activity of the FLE mixture also varied depending on the time of assay. Experiments take time, and all the assays for measuring ATP in an experiment are obviously not accomplished simultaneously, but sequentially. If enzyme activity diminishes as shown in Figure 5, then comparison of assays accomplished 1-2 hours apart introduces error. Figure 6 conclusively shows that this type of error was not inherent to the protocol used for these studies because the B/A ratio was determined as a measure of the ATP in an unknown.

Accuracy of the measurement of ATP was further refined by providing for the greatest enhancement of the signal practicable. Quenching due to glass cuvettes was documented (Figure 7) and was overcome by using plastic cuvettes. Consequently, the absorption of photons of light by the glass, a source of error peculiar to counting with a PMT, e.g., in liquid scintillation counting, was minimized.

Likewise, the assay signal was enhanced by the addition of excess luciferin in the form of a synthetic molecule. This resulted in an intensification of the assay signal above the level

of the background signal at concentrations above 10 nM and, on a molar basis, provided a 10-fold greater degree of detection of ATP (Figure 8).

Based on the evidence presented, it is concluded that the techniques employed and precautions taken could not have provided for an accurate and precise measure of ATP under the experimental conditions. The assay would have detected an ATP difference between samples of one nanomolar. ATP would not have been confused with another nucleotide because of its distinctive signal characteristics and the standard dose-response curve. It is recommended that these techniques be employed in the study of ATP whether or not commercial or specially-designed equipment is utilized.

Frog Heart Perfusion

Without a coronary circulation to influence the delivery of oxygen or the removal of metabolites in the in situ frog heart, it was thought that a study of the release of ATP, if any, would be rather straightforward. Several considerations deserve mention.

First, a perfusion pressure of 90 mm H₂O was found through trial and error to provide adequate venous return to the heart. A proper size and length of tubing was chosen to provide an adequate but not excessive peripheral resistance. Cardiac output was not a maximum and a Frank-Starling effect could be produced to demonstrate the vitality of the heart.

One of the possible influences on the accurate measurement of ATP release from the frog myocardium was an ionic effect on the FLE. Light emission is diminished linearly as the log of the cation concentration increases (10). The influence of the cation concentration on light emission was verified by constructing standard curves similar to Figure 4, but with varying osmolarity. Light emission was slightly less when osmolarity was increased. Since ionic exchange occurs in the perfusate going through the frog heart, it was thought that the ATP levels would be underestimated. Unfortunately, the opposite was shown to occur for other reasons.

ATP levels were elevated due to the presence of red blood cells. Since the majority of the perfusion experiments were conducted without consideration of this possibility, it is not possible to say with absolute certainty that Figure 15 is representative of what occurs in frog myocardium during a hypoxic or hyperoxic insult. Additionally, it is not possible to exclude the release of ATP from the red blood cells once they have come into contact with the hypoxic or hyperoxic perfusate (see DISCUSSION on ATP Efflux From Erythrocytes).

Finally, it may have been a consequence of red cell contamination, rather than a basal level of myocardial release of ATP, that resulted in no measurable ATPase activity when 1 nM ATP was perfused through the frog heart (Figure 17). Again, these experiments were conducted prior to any understanding of a red cell contribution to ATP or ATPase activity.

RBC Protocol

The first methodological consideration in the study of the frog red blood cells was that the red cell samples were not pure, but contained some white blood cells and spindle cells (platelets). No separation procedures were employed to segregate the various blood elements from each other. Instead, whole blood was harvested and then diluted with frog Ringer's solution approximately 1000 times. A count of the red cells, the platelets, and the white cells would also show a 1000-fold dilution into the range of 400, 4, and 3 cells per microliter, respectively.

This was partially verified by counting the red cells and spindle cells. Such counts constituted a major effort in this protocol. Spindle cells were routinely counted because they could be easily differentiated. White cells were not distinguishable from red cells unless stained. For this reason they were not counted, and possibly were included in the red cell count. Based on their constituency in whole blood (384), a dilution to 3 cells per microliter was assumed.

A primary consideration in the harvesting and treatment of the red cell samples was that they were never centrifuged, washed with any drug or enzyme, or mishandled (shaken, stirred, etc.). In principle, then, the cells should not have been lysing. Consequently, considerable effort was made to determine the mechanism of release.

One possibility for the cause of ATP release that depended on methodology is permeabilization. Red cells and other transformed cells can be permeabilized with ATP at concentrations exceeding 100 μM (205,328,370,454). This allows large molecules, including intracellular ATP, to efflux from the cells (see HISTORICAL REVIEW). It is uncertain what the circulatory level of ATP was prior to cell harvest. To what degree surgery elevated plasma levels of ATP prior to cell harvest, resulting in permeabilization, was also unknown. The nanomolar level of ATP found in all dilute cell samples (from 80 to 1200 cells/ μl) may have been indicative of a normal plasma level of ATP in the micromolar range, since the whole blood was diluted 1000 times. On the other hand, if a normal circulating level of ATP was less than micromolar in concentration, then the trauma of surgery may have added significant levels of ATP and/or the frog red cells may have been permeabilized at an ATP concentration less than 100 μM . This is the concentration required to permeabilize dog red cells (328).

Permeabilization would then have been responsible for the results observed with settled cells. If the cells had become permeabilized prior to harvesting, and remained so, then once they had settled the local concentration of ATP may have increased, exacerbating the permeabilization. This was only a remote possibility, however. For other reasons it is thought that these red cells were releasing ATP for a physiological purpose (see DISCUSSION on ATP Efflux From Erythrocytes).

Stirring the cells by keeping the plastic microvials on a tube rotator was originally thought to be harsher treatment than allowing the samples to settle. It was postulated that movement of the cells would infrequently, but with some certainty, cause contact with the side of the microvial and result in cell lysis. ATP concentration was expected to rise subsequently. Neither postulate was confirmed. Rather, counting the samples before and after rotation on the tube rotator provided no statistical basis for cell lysis. The ATP concentration did not increase either (see Figure 27). Of course, this may have been due to the ATPase activity of the cells. An additional marker, such as lactate dehydrogenase or hemoglobin, would have helped to verify if lysis was occurring.

Counting the cells by hemocytometer was a routine procedure, described in METHODS. It was based on the practice followed in most blood banks, but specifically that at the St. Louis University Medical Center.

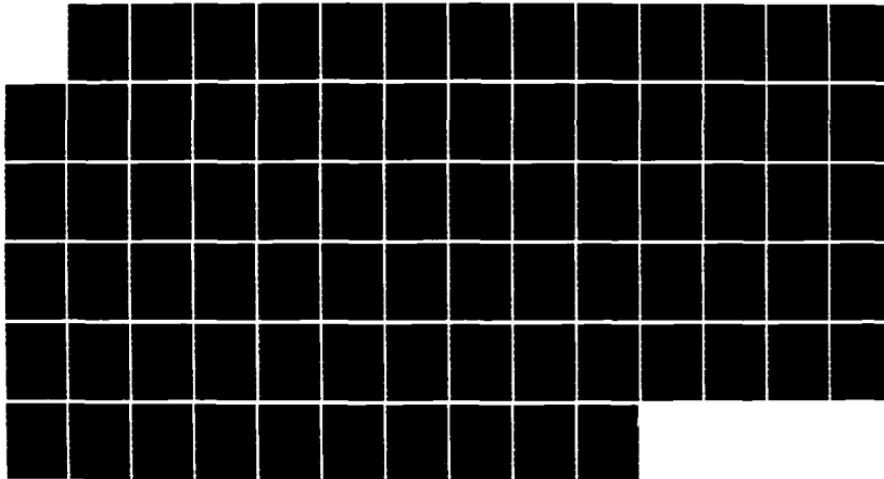
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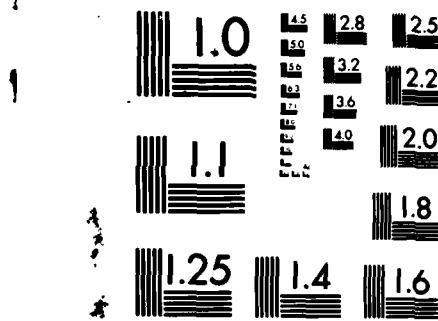
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Nucleated red blood cells from salamanders were also briefly studied with the intent of verifying any similar release of ATP as found in frog red cells. As described in the RESULTS, the effort proved futile because of the size of the cells. The injection port of the sample holder used to assay for ATP did not accept a hypodermic needle larger than 20 gauge. The salamander cells were too large to be aspirated through anything less than possibly a 15 gauge needle. Experience with the 20 gauge needle showed that several successive assays of the same cell sample over a brief period of time produced highly variable levels of ATP. It was concluded that the salamander cells were lysed as they passed through the needle.

DISCUSSION PART II: RESULTS

ATP Release From Frog Myocardium

Langendorff-perfused hearts (guinea pig) (326,421), working hearts (rat and frog) (82,124) and isolated myocardial cells (rat) (151,463), have been shown to release ATP. Hypoxia was hypothesized to be the signal. In seven of fifteen frog hearts, ATP appearance increased upon exposure to hypoxic frog Ringer's solution. However, the supposed cell-free effluent from the hearts produced considerable variability during the test protocol. The result was that no statistical difference could be shown between control and hypoxic conditions. The same statement can be

made for effluent samples collected under hyperoxic conditions. Each effluent sample was collected with the expectation that the ATP entering the solution upon one pass through the frog heart could be kept on ice without any effect on the ATP concentrations. All samples were observed to be clear at the time of collection, but many samples were observed to have erythrocytes settled at the bottom when left untouched for 20-30 minutes or longer. Initially the concern had been with ATPase activity. If any change were to occur on the ATP concentration of the effluent samples, it was expected to be a decrease. Consequently, ATPase activity by the red cells would have produced an underestimation of the amount of ATP release from the frog heart (438).

Though it was only subjectively noted, the appearance of the red cells in the effluent appeared to be greater when the work of the heart increased. Since an increase in work, as measured by cardiac output, occurred upon exposure to a perfusate with low or high pO_2 , a greater number of cells appeared in the effluent and affected the results. Based upon the subsequent study of the ATP and the ATPase activity associated with frog red cells, in retrospect it seems reasonable to assume that the red cells were a major cause of the extreme variability seen in the release of ATP from frog myocardium.

The source of these erythrocytes was thought to be other blood vessels emptying into the posteror vena cava. Thus, diastolic recoil during increased cardiac output was suspected of enhancing venous return vis a fronte, and consequently increasing the appearance of red cells in the effluent.

When erythrocytes were released into the perfusate going through the heart, a dynamic situation arose that depended on the number of cells present. Initially the cells hydrolyzed ATP. Thus, the ATP concentration decreased. However, when the cells settled by gravity, they were then a source of ATP. The protocol for the study of myocardial ATP release, when initiated, did not take this into account.

Likewise, other studies involving ATP release from heart or skeletal muscle must be re-examined in light of these findings. Although anucleate erythrocytes have not been studied to show a similar association of ATP, virtually all studies conducted with amphibians or other species with nucleated red cells must account for ATP from ethrocytes. Not only may they function as a source for ATP, but ATP release may be caused by some drug or other substance intended to act only on the endothelium or the muscle. If ATP from red cells is present, then activation of a P_2 receptor on some cell may affect the results of the research protocol. The study of hyperemia would be particularly vulnerable to errors if erythrocytes were present.

ATPase Activity of Frog Myocardium

The level at which ATP appears in circulation in the frog heart appears to be in the nanomolar range. When ATP was perfused through the heart, substantial hydrolysis of ATP should have occurred not only at 10 nM and above, but also when 1 nM ATP was perfused through the heart. This did not happen. Instead, ATP was added to the perfusate.

The source of this ATP may have been myocardial cells, nerve cells, the littoral cells lining the trabeculae (i.e., endothelial-like cells), or possibly blood elements. The solutions passed through the heart were normoxic, and therefore should not have activated a hypoxia-dependent release mechanism in any of the tissues. Except for reasons of cell injury, one does not ordinarily expect to see ATP appear outside of cells. Under these conditions, why did ATP increase in the perfusate containing 1 nM ATP?

A small, basal level of ATP release from cells does not seem implausible if a mechanism exists for the movement of the ATP molecule across the bilipid cell membrane. Options for moving ATP across the plasmalemma include a channel and/or a carrier-mediated translocator, for which no evidence currently exists. Exocytosis, another option, is more applicable to a secretory or neurosecretory event, and also seems unlikely. "Nucleotide permeabilization" is not applicable because the criteria for it are not satisfied.

The mechanism of release of ATP from myocardial cells in response to hypoxia may be hypothesized to contribute to some, if not all of the ATP seen in the effluent. Although no evidence has been presented for a nucleotide translocator protein in the cell membrane, its presence may exist if only as a consequence of cells' production of mitochondrial protein responsible for ADP-ATP translocation (16,41,133,254,255,256). Consequently, if the process for protein selection and insertion into mitochondrial membranes were less than 100% efficient, then the appearance of the ADP-ATP translocator protein in the sarcolemma might be possible. This hypothesis has never been documented.

A more probable option, which is more easily defended, is that of a channel. Such a mechanism for release of ATP may have specific characteristics which are sensitive to manipulation. Again, no evidence for an ATP anion channel in cardiomyocytes has been documented, nor was any evidence for such produced in this research.

Purinoceptor-Mediated Calcium Mobilization

Receptor-mediated calcium mobilization has been partially disclosed in the frog heart (398). Epinephrine is a positive inotropic agent that modulates intracellular calcium via the cAMP pathway. ATP apparently can enter into this regulatory system through the cyclic nucleotide pathways (140). This is thought to contribute to the positive inotropism seen following the application of ATP. Indomethacin (10 μ M) also was shown to

diminish the first phase and abolish the third phase of the response, thus implicating prostaglandins in the mobilization of calcium (140). The data hereby presented on the enhancement of calcium mobilization by ATP following a brief exposure to either of two calcium blockers indicated that

- 1) the P_{2X} receptor was probably not associated with the voltage-sensitive calcium channel responsible for tension development, and
- 2) at least some of the calcium responsible for the first phase of the triphasic response originated extracellularly.

The reason for the first conclusion was because the calcium blockers did not block the P_{2X}-mediated calcium mobilization. Instead, the blockers enhanced it. This contrasts with what is known about their blocking action, i.e., calcium is prevented from moving through the voltage-sensitive gate (47,388). Apparently more free calcium moved down its gradient through a receptor-mediated channel when these blockers were present.

It is confusing that these calcium blockers were able to enhance the ATP effect (P_{2X} activation). The manganese action was likely a consequence of the displacement of a significant amount of calcium from external binding sites (274). This raised the local concentration outside and increased contractility during P_{2X} activation. It was the displaced calcium, therefore, that was suspected of enhancing the P_{2X} calcium mobilization seen in Figure 19. That ATP also affected the action potential (140,320) is probably an event separate from the receptor-mediated

calcium entry. Either that, or ATP was in some way able to overcome or use to its advantage the binding of the calcium blockers to the voltage-dependent channels. If this were so, the combination of blocker and ATP likely functioned as a calcium channel activator, e.g., BAY K 8644 (388).

Such a complicated hypothesis is only entertained because of the results with verapamil. Verapamil does not displace calcium, but rather antagonizes its influx by binding to a site on the channel (231,274,312,388,408). In this capacity it is a "use-dependent" blocker. For voltage-dependent channels, access to verapamil's binding site occurs when depolarization opens the channel. Were verapamil to function in the same manner for a P₂X-mediated channel, it would also block the action of ATP. Figure 20 shows that it enhanced, rather than blocked, the action of ATP.

Does this indicate that ATP and verapamil together functioned like a calcium channel activator? A simpler explanation is suggested. Verapamil may have bound to and in some way enhanced calcium movement through a P₂X-mediated channel. By modulating the conformational shape of a protein in the channel, verapamil may have allowed greater calcium influx when the channel was opened by ATP. Perhaps other protocols using dihydropyridines or calcium channel activators will provide evidence for the source of this calcium.

The findings with these blockers also allowed the conclusion that at least some of the calcium entered from the outside of the cell. Any other conclusion would have to explain how these so-called antagonists entered the cell to facilitate calcium release from the SR, the other proposed origin of calcium mobilized by ATP (320).

The attempt to localize the calcium mobilized by ATP included an effort to completely antagonize the effect using a new P₂ blocker. No reversible P₂ antagonist presently exists, and the findings with Isatogen 151-3 were only sufficient to conclude that further research is warranted. The trend, based on three animals, is that this substance may be a P_{2X} blocker.

ATP Efflux From Frog Erythrocytes

Based on the assays of the perfusate from frog hearts, it became apparent that frog erythrocytes were influencing ATP levels. The assay procedure was able to measure ATP at a sensitivity of from 1 nM to about 2500 nM, allowing the study of very dilute cell samples. When harvested and diluted about 1000 times, the erythrocytes from all the frogs consistently presented with extracellular ATP levels near 10 nM.

This may have been due to the release of ATP from the erythrocytes in circulation. Experimental results showed that cells allowed to settle for over a half hour appeared to release ATP. Perhaps the same thing happens in circulation during

stagnant circulatory flow, or during hypoxia. If ATP is released from these red cells in vivo, two physiological events are expected:

- 1) increased oxygen uptake and delivery, and
- 2) increased blood flow due to an ATP-mediated endothelium-dependent vasodilation.

In the nucleated red cells of fish, eels, and amphibians, ATP functions as more than a source of energy. It is also an organophosphate that plays a major role in controlling the affinity of hemoglobin for oxygen (23,200,455,470). ATP acts similarly to 2,3-DPG in its allosteric control over hemoglobin. Both decrease hemoglobin's affinity for oxygen. The difference between them is the manner by which they are utilized by the red cells of the host animal. Human erythrocytes make additional 2,3-DPG when exposed to hypoxia (277). Red cells of aquatic animals decrease their level of ATP (470). This latter phenomenon is critical to the increased uptake of oxygen from the hypoxic water environment. Oxygen-carrying capacity is, in turn, increased.

During hypoxia, the decrease in ATP in red cells of fish, eels, and amphibians has been attributed to a decrease in the enzymes associated with ATP production, specifically oxidative phosphorylation (174,351,470). The result, as first reported by

Wood and Johansen (470), is a leftward shift in the oxyhemoglobin dissociation curve and a decrease in the P_{50} . This causes an enhanced oxygen carrying capacity that benefits the animal.

Based on the results of experiments with frog erythrocytes, the hypothesis must also be entertained that the nucleated red cells decrease their intracellular ATP via efflux through an anion channel. This release of ATP, as seen in these experiments, was probably in response to hypoxia. It occurred acutely, since cells allowed to settle for 50-60 minutes demonstrated ATP efflux. Stirred cells did not. It is suggested that the settled cells experienced localized hypoxia in the bottom of the microvial because they used the surrounding oxygen. The decreased oxygen provided the signal for the release of ATP from the red cells.

Nucleated red cells consume almost 10 times more oxygen than do anucleate red cells (389). Therefore, it is reasonable to conclude that local oxygen levels immediately around the settled cells diminished accordingly. The cells then responded to this by releasing ATP. In contrast, cell samples maintained on a tube rotator kept the cells in a normoxic environment and prevented localized hypoxia.

ATP efflux from frog cells was seen within one hour. Hypoxia-induced reduction of ATP within one hour has also been documented in the red cells of Fundulus heteroclitus (175) and

Squalous acanthius (455). The time course of the decrease in intracellular ATP observed in these latter two instances correlates extremely well with the time course of the increase in the extracellular ATP seen with frog red cells. Therefore, hypoxia-induced reduction of ATP in nucleated red cells most likely is a consequence of both an increased efflux of ATP and diminished oxidative phosphorylation.

One effort to make the frog red cells hypoxic in order to verify this hypothesis was not productive. Bubbling with 5% CO₂-95% O₂ only lysed the cells. Other procedures have yet to be tried.

Another experimental technique utilized to try and cause ATP efflux from frog erythrocytes was depolarization with either high potassium or veratridine. White (458,459) was able to depolarize synaptosomes with these agents in front of a photomultiplier tube and demonstrate the release of ATP. Since both human and amphibian red cells have a transmembrane potential (213), it seemed reasonable to try to cause ATP release with this technique in frog red cells.

Whether or not the cells were depolarized was not ascertained through any electrophysiological means. Though depolarization was likely with 40 mM potassium, no increase in the light signal was observed. This concentration of potassium may have diminished the firefly sensitivity (10). Veratridine (50 μ M) caused no increase in the light signal either. Thus, ATP efflux could not be induced

by depolarization, and hypoxia-induced release of ATP is not likely to be the result of a physical alteration in the membrane during a change in the transmembrane potential.

Vasodilation produced by EDRF release from the endothelium in frog has not been documented. However, in all probability this type of vasodilation exists. If ATP efflux from red cells does occur in vivo, ATP would mediate a hyperemia independent of the metabolism of the surrounding tissues. That is, a hyperemia not tied to the release of a metabolic factor, but to a decreased partial pressure of oxygen in the blood, would result.

It is thought that the release of ATP is best characterized as an efflux through an anion channel. Though the concentration difference from inside to outside the cell is over 1000, ATP is not lipid-soluble and its diffusion coefficient is such as to preclude simple diffusion. That leaves facilitated transport, either by a mobile carrier or a channel, as the likely efflux process.

Facilitated transport exhibits saturation kinetics and is influenced by specific inhibitors. Mobile carriers have a high activation enthalpy and a low turnover number, or conductance. Channels, on the other hand, may or may not be temperature sensitive, but the turnover number is usually high.

If a mobile carrier were responsible for the release of ATP, then it would be temperature sensitive. An early approach in the study of red cells included placing the cell samples on ice. Unfortunately the time between assays at this stage of the study was too brief (< 20 minutes) to cause ATP release, and in turn, to see if temperature decreased the release. ATP efflux was induced most effectively by allowing the cells to settle for about one hour. Since this was not done with samples on ice, a comparison of the effects of temperature on ATP efflux was not possible.

The number of efflux "pathways" in the plasmalemma of the frog red cells is obviously not known, and for this reason it is not possible to calculate the conductance of ATP per pathway. Nevertheless, the efflux values shown in Figure 28 are comparable to a release of about 40,000 molecules per cell per minute. This suggests a channel, rather than a mobile carrier, as the type of facilitated transport mechanism that is at work. Evidence with anion channel blockers seems to support this suggestion.

ATP Efflux Inhibition: Anion Channel Blockers

Specific inhibitor substances are frequently shown to impede facilitated transport. In mitochondria, for example, atractyloside or bongrekic acid each have their own unique way of blocking the transport of ATP (16,256). With regard to red cells, pyridoxal phosphate, probenecid, 4-acetamide-4'-isothiocyanato-2, 2'-disulfonic stilbene (SITS), prostaglandin A₁, nitrobenzyl-thioinosine (NBFI), ouabain and furosemide have been used to

inhibit the transport of various ions, nucleosides (adenosine), or nucleotides (cAMP) across the cell membrane (66,105,201).

In these experiments pyridoxal phosphate caused inhibition of the firefly enzymatic reaction. Ouabain and SITS were tried without effect, possibly because the time between assays was too short (< 20 minutes between each) and did not allow the alleged hypoxia-induced mechanism to work effectively. Three substances, though, including atractyloside, probenecid and furosemide, were used with varying degrees of success to limit the efflux of ATP from frog red cells.

The efflux inhibition by probenecid (Figure 30) may indicate that all nucleotide translocation in nucleated cells occurs via a similar process. Cyclic AMP efflux from avian red cells is probenecid-sensitive (68), and the present results show that ATP efflux is probenecid-sensitive. The movement of these two nucleotides may traverse the cell membrane via the same or similar channels. Comparison of cAMP and ATP efflux in frog cells with that in pigeon cells may prove beneficial to establishing commonality of the release mechanism. Also, a functional purpose for the release of these nucleotides may be uncovered. Whereas ATP decrease in red cells of aquatic animals is known to benefit oxygen delivery, the meaning of cAMP efflux in avian red cells remains obscure.

The effects with furosemide generate further questions.

Furosemide, an anion (chloride) channel blocker, is less effective than probenecid, but is able to decrease ATP efflux from frog red cells (Figure 31). This may indicate, contrary to evidence with cAMP efflux in pigeon red cells (201), that ATP efflux from frog cells may be sensitive to chloride movement across the plasmalemma. ATP is charged, and its movement from one side to the other entails an imbalance in negative charges that must be corrected if the transmembrane potential is to be maintained.

There are two possibilities. First, that the efflux of ATP is furosemide-sensitive seems to suggest that the negative charges of chloride may be needed inside in exchange for the loss of the negative charges of ATP. If chloride influx is blocked by furosemide, then ATP efflux is diminished.

Second, hypoxia in these red cells portends a decrease in the intracellular pH. Lactic acidosis accompanies the decrease in intracellular ATP seen in eel erythrocytes (470). A concomitant movement of hydrogen ions out of the cell may occur with the movement of the negative charges of ATP out of the cell. Hydrogen ions may bind to ATP and cancel the negative charges, allowing the movement of an uncharged molecule across the plasmalemma. In this way, the transmembrane potential is not disrupted.

Additional studies are warranted in order to provide an understanding not only of charge distribution but also nucleoside-nucleotide balance. Concerning the latter, a loss of ATP requires replacement of the purine component. De novo synthesis or, perhaps adenosine influx, is required. If adenosine uptake is required, maybe the channel is an antiport, moving adenosine in and ATP out. Results using SITS and NTBI to further describe the specificity of this channel for anions and nucleosides would be beneficial.

Though probenecid was an effective antagonist, no exclusive traits are ascribed to the anion channel responsible for ATP efflux. It may be that the channel can transport other organic anions in the same manner as in renal tissue. Thus, the probenecid-sensitive transport of p-aminohippurate (PAH) and adenine nucleotides described by Maxild (290) in the kidney also may be characteristic of the frog red cell. If this is true, then ADP and AMP may compete with ATP. Otherwise the release of ATP is a specific response that has little to do with ADP and AMP movements.

One reason that renal tissue and red cells may not necessarily be comparable in this regard is that the movement of ATP occurs in opposite directions. That in the kidney slices was an uptake against an electrochemical gradient, implying perhaps, a mobile carrier similar to that seen in other tissues (1,261,275,281,282). That in the red cell is obviously movement

down the electrochemical gradient of ATP at a rate that signifies, in all probability, a channel. Further study is necessary in order to determine if this anion channel is nucleotide-specific.

With regard to the process of "nucleotide permeabilization", two pieces of evidence were gathered that support conflicting arguments. Permeabilization was not seriously considered as the cause of ATP efflux because none of the criteria for it fit the experimental circumstances described herein. Among the criteria for permeabilization are an alkaline pH, absence of glucose, and extremely high ATP (100-250 μ M) (205). Unpublished observations of G. Weisman (see 205) show that furosemide and bumetanide inhibit permeabilization. Thus, data on furosemide in this study may support permeabilization.

Alternatively, trifluoperazine (TF) has been shown to exacerbate the permeabilization process in transformed cells (205,253). In one experiment with frog red cells, however, TF at 5 μ M and 10 μ M inhibited ATP efflux. Higher concentrations caused cell lysis, as determined by hemocytometer counting. The opposite should have occurred, and therefore permeabilization was not supported by these data.

It also is possible that the concept of permeabilization is improperly characterized as non-physiological. Nucleotide permeabilization as described by others may have resulted from more than one membrane event and caused erroneous conclusions. If

ATP efflux through an anion channel was occurring in these protocols, there was no way to determine it because of the massive exogenous levels added to the cells.

The evidence with atractyloside shown in Figure 32 supports the hypothesis of efflux by way of a facilitated transport through a channel. Like simple diffusion, movement through a channel is dependent on the concentration gradient. If this gradient is diminished by incubating the cells with atractyloside, a decreased efflux will likely occur.

Intracellular ATP of Amphibian Erythrocytes

The efflux of ATP from these red cells raised another issue. The extracellular ATP that was measured undoubtedly constituted some percent of the intracellular pool of ATP. Determining this percentage was relatively easy, but the interpretation proved otherwise.

Two of the methods used to lyse the frog red cells (sonication and digitonin) provided reasonable results. Approximately 3 mM ATP is comparable to human $[ATP]_i$ and approximates previous observations with frogs. The $[ATP]_i$ determined in salamander red cells with digitonin appears low by comparison to the frog. No other value has been published on $[ATP]_i$ of salamander red cells.

The assumptions used in the calculation of $[ATP]_i$ did not address the issue of bound, or structural, ATP. The firefly bioassay method measured free ATP, not ATP bound to or sequestered within protein structural components of the cell. For this reason, then, all of the ATP in the red cells may not have been measured. Some ATP may have remained bound. If this is true, then the conclusions about $[ATP]_i$ may be in error.

Lysing the cells in front of the photomultiplier tube was a process that did not release all of the free ATP at the same time. Consequently the height of the recorded light signal was not an accurate measure of all the ATP released, because not all of the ATP was present at the same time to generate the signal. This probably accounted for the low average $[ATP]_i$ seen using this method (see p. 166).

If 2.9 mM is an accurate estimate of the intracellular ATP of frog cells, then each cell contains about 1.5 billion molecules of ATP. The efflux of ATP from these cells was reported in molecules/cm².sec in the Figures. Another way to report efflux is in molecules/cell·min. For frog cells, the efflux rate was about 40,000. This is about 0.027% of the intracellular ATP, a figure that seems numerically small but when released to the cell's exterior is extremely potent as a vasodilator.

This degree of loss of ATP from the interior of the cell may appear to be negligible vis-a-vis altering the affinity of hemoglobin for oxygen. Greaney and Powers (175) did not report raw data in Fundulus heteroclitus. Instead, graphic data appears to show that a one hour exposure of these red cells to an anoxic medium decreased the mole-to-mole ratio of ATP to hemoglobin by 25%. Since the amount of free and bound ATP is not known, an attempt to relate the amount effluxing per minute to the amount required for allosteric binding to hemoglobin is not possible.

The release of such a small percentage of ATP from the intracellular compartment also may reflect a physical effect caused by the loss of serum proteins from around the cells. The method for studying these cells required dilution of whole blood, with dilution of the plasma proteins also occurring. Specifically, albumin was no longer layered around the cells.

Research has shown that increased deformability of the red cell membrane occurs with decreasing protein adsorption (249,250,462). A loss of plasma proteins during dilution may have significantly affected normal membrane function, resulting in the appearance of ATP during these protocols. This explanation is arguably weak, though, in light of the results with probenecid, furosemide and atractyloside.

Erythrocyte Ecto-ATPase Activity

Exogenous ATP added to frog erythrocytes was shown to be hydrolyzed, as seen by a decrease in the extracellular ATP concentration over time (Figure 29). The rate of disappearance of ATP was described mathematically as an exponential curve. Determining the true rate of ATP hydrolysis by the ecto-ATPases depended on what part of the curve was chosen to make the calculation.

Ideally, the assays should have been accomplished every 30 seconds in order to determine the tangent line of the curve early in the hydrolysis. This was not possible. Instead, an exponential regression of the curve was determined and used to provide an estimate of these points. The difference in the assays between time zero and 4 or 5 minutes was then used to calculate the hydrolysis rate.

The average rate was 2.3×10^7 molecules/cell·min. This indicates that an excess ATPase activity per cell exists. Since 100-times less ATP per cell was observed to efflux to the outside, the ATP levels cannot be described as too high. During this protocol, ATP efflux was always less than the cells' ability to hydrolyze it.

The ecto-ATPases have been reported to be inhibited by EDTA (443), but this was not seen in these experiments. EDTA had no

effect on the rate of ATP hydrolysis. Others have used 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, or EDAC, to inhibit ecto-ATPases with some success in D. discoideum (327), but this was not tried with frog red cells.

The ability of frog red cells to hydrolyze ATP also may have prevented accurate characterization of the ATP efflux. A major consideration was that when red cell samples were maintained on a tube rotator, i.e., the cells were stirred, the extracellular ATP concentration had a tendency to decrease. This is an indication of the dynamic situation that exists in the study of the extracellular ATP associated with amphibian erythrocytes. When cells were not moving, as with settled cells, the hydrolytic rate was so low that the external milieu of the cell increased its ATP concentration. If the cells were moving such that ATP came into contact with their ecto-enzymes, then hydrolysis occurred. The question not answered was whether the stirred cells also released ATP at some basal rate.

ATPase hydrolysis may have prevented accurate measurement of $[ATP]_i$. Lysed cells were not diluted and stirred as were whole cells. Consequently, any residual activity of the enzyme protein following lysis was not determined. If it existed, the procedure for determining $[ATP]_i$ would have underestimated it.

It is difficult not to address the existence of extracellular ATP and ecto-enzymes in a teleological way. That is, if red cells are not expected to see ATP at a relatively high concentration, then why are ecto-ATPases integral to their membrane? It may be that, as a routine matter, these cells hydrolyse more than just the ATP that appears through injury and trauma, cell death, etc. ATP may be released because of localized hypoxia. Also, cell-to-cell communication may occur via ATP release under conditions not presently appreciated but nevertheless requiring a process for keeping it under control.

SUMMARY

Documented in this series of experiments are results showing that ATP can and does move across the plasmalemma to the cell exterior. This idea originated with Thomas Forrester with regard to functional hyperemia and has been a source of contention for many years. Obviously, understanding of it has been lacking. Perhaps the nucleated red cell can function as a limited model for release of ATP from other cells, e.g., myocardial cells.

This research effort was originally intended to document a release of ATP from the frog myocardium in response to a hypoxic challenge. Instead, evidence was found implicating red cells as a major source of ATP contamination. A study of ATP release from frog myocardium was near to impossible unless red cell contamination could be solved. This problem remains.

Hypoxic and hyperoxic frog Ringer's solution increased ATP release from the frog heart. Unfortunately, the red cells were found to constitute more than just a minor source of ATP. Frequently, red cell ATP surpassed that allegedly contributed by the myocardium.

The ATP associated with frog erythrocytes could be controlled to varying degrees by keeping the cells stirred or exposing them to substances that appeared to inhibit ATP efflux. Lysis was refuted as a cause for the ATP observed near the cells. Instead,

the layering of ATP in the supernatant above settled cells, in contrast to observations with stirred cells, gave rise to the hypothesis that these nucleated red cells were becoming hypoxic. Physiologically, the cells responded by releasing ATP to the outside. If it indeed can occur in vivo, this cellular response is expected to enhance oxygen delivery and increase blood flow.

Questions abound and only further research will answer them. Is hypoxia the real cause of ATP release? Does ATP release from these cells occur at some basal rate? Is an anion channel the proper characterization of the release mechanism? Is this release mechanism specific for ATP? How does ATP release affect other ion movements and the transmembrane potential of the cell? Does this amount of ATP release cause a leftward shift of the oxyhemoglobin dissociation curve? Does an endothelium-dependent vasodilation occur in vivo and result in increased blood flow and oxygen delivery? These questions and others await the inquiring mind.

APPENDIX I

ATP concentrations in red blood cells are normally reported as the following:

- a) millimoles ATP per 1000 ml packed cells
- b) micromoles ATP per gram hemoglobin
- c) micromoles ATP per 1000 ml whole blood
- d) picomoles ATP per number of cells

To convert these values to Molar (moles/liter), the following procedures were used:

For humans, assume:

- 1) hematocrit of 47% (12)
- 2) mean cell volume of $94 \mu^3$ (12)
- 3) 96% of a packed cell volume is cells, and 4% is interstitial water (178)
- 4) mean cell hemoglobin concentration of 33.5 gm per 100 ml RBC, or 34.9% (12)
- 5) 65% of the red blood cell is water (114)
- 6) RBC count of 5.40×10^6 cells/ μ l (12), or 1.15×10^7 packed cells/ μ l.

For frogs (Rana pipiens), assume:

- 1) hematocrit of 40% (193)
- 2) mean cell volume of $800 \mu^3$ (193)
- 3) 96% of a packed cell volume is cells, and 4% is interstitial water (178)
- 4) mean cell hemoglobin concentration of 10.4%, or 10 gm per 100 ml RBC (193,276)
- 5) 65% of the red blood cell is water (114)
- 6) RBC count of 0.48×10^6 cells/ μ l, or 1.2×10^6 packed cells/ μ l (193)
- 7) no adjustment for nuclear volume (Though the nucleus of the frog red cell (194,272) may equal 20% of the cell volume, no adjustment for nuclear volume was made because the concentration of hemoglobin, and presumably water and ATP in the nucleus is equal to that in the cytoplasm (103).)

APPENDIX I (CONT.)

If red cell ATP is reported as 0.9×10^{-3} moles/liter cells (461), then

$$\begin{array}{c}
 \frac{0.9 \times 10^{-3} \text{ moles}}{1000 \text{ ml of cells}} \times \frac{1}{X} = \frac{0.96}{X} \\
 \\
 \frac{1 \text{ ml}}{1000 \mu\text{l}} \times \frac{1 \mu\text{l}}{5.4 \times 10^6 \text{ cells}} = \\
 \\
 \frac{1 \text{ cell}}{94 \times 10^{-15} \text{ liters}} \times \frac{1}{X} = \frac{0.65}{X} \\
 \\
 = 2.84 \text{ mM ATP per cell}
 \end{array}$$

If red cell ATP is reported as 4.5 micromoles/gm Hb (37), then

$$\begin{array}{c}
 \frac{4.5 \times 10^{-6} \text{ moles}}{\text{gm Hb}} \times \frac{335 \text{ gm Hb}}{1000 \text{ ml cells}} \times \frac{1}{X} = \frac{0.96}{X} \\
 \\
 \frac{1 \text{ ml}}{1000 \mu\text{l}} \times \frac{1 \mu\text{l}}{5.4 \times 10^6 \text{ cells}} = \\
 \\
 \frac{1 \text{ cell}}{94 \times 10^{-15} \text{ liters}} \times \frac{1}{X} = \frac{0.65}{X} \\
 \\
 = 4.76 \text{ mM ATP per cell}
 \end{array}$$

APPENDIX I (CONT.)

If red cell ATP is reported as 433 micromoles/1000 ml whole blood (39), then

$$\begin{array}{rccccc}
 433 \times 10^{-6} \text{ moles} & & 1 & & 1 & \\
 \hline & X & \hline & X & \hline & X \\
 1000 \text{ ml whole blood} & & 0.45 & & 0.96 & \\
 \hline & & & & & \\
 1 \text{ ml} & & & & 1 \mu\text{l} & \\
 \hline & X & & & \hline & X \\
 1000 \mu\text{l} & & & & 5.4 \times 10^6 \text{ cells} & \\
 \hline & & & & & \\
 1 \text{ cell} & & & & 1 & \\
 \hline & X & & & \hline & = \\
 94 \times 10^{-15} \text{ liters} & & & & 0.65 & \\
 \hline & & & & & \\
 = & 3.04 \text{ mM ATP per cell} & & & & \\
 \end{array}$$

If red cell ATP is reported as 1306 picomoles/ 10^7 cells (108), then

$$\begin{array}{rccccc}
 1306 \times 10^{-12} \text{ moles} & & & & & \\
 \hline & & & & X & \\
 10^7 \text{ cells} & & & & & \\
 \hline & & & & & \\
 1 \text{ cell} & & & & 1 & \\
 \hline & X & & & \hline & = \\
 94 \times 10^{-15} \text{ liters} & & & & 0.65 & \\
 \hline & & & & & \\
 = & 2.14 \text{ mM ATP per cell} & & & & \\
 \end{array}$$

Intracellular ATP using original research data was calculated as follow:

$$\begin{array}{rccccc}
 [\text{ATP}]_i = & \text{moles/liter} & & 1 \text{ cell} & & 1 \\
 & X & & \hline & X & \hline \\
 & N & & 8 \times 10^{-13} & & 0.65 \\
 & (\text{cells/liter}) & & \text{liters} & & \\
 & & & (\text{cell volume}) & & \\
 & & & & & \\
 = & \text{mM ATP per cell} & & & & \\
 \end{array}$$

APPENDIX II

ATP hydrolysis was either reported directly as molecules per cell·min or was calculated as follows:

1) assume the following hematocrits and erythrocyte counts per microliter:

			reference
eel--	37.9%	2.48×10^6	(12)
frog--	40.0%	0.48×10^6	(193)
rabbit--	41.5%	5.70×10^6	(12)
cat--	40.0%	8.00×10^6	(12)
man--	47.0%	5.40×10^6	(12)
rat--	46.0%	8.90×10^6	(12)

Then, packed cells per μl are the following:

eel--	6.54×10^6
frog--	1.20×10^6
rabbit--	1.37×10^7
cat--	2.00×10^7
man--	1.15×10^7
rat--	1.93×10^7

If ATP hydrolysis is reported for the frog (438) as 37 nmol/ml cells·min, then

$$\begin{array}{rccccc}
 37 \times 10^{-9} \text{ moles} & & 6.02 \times 10^{23} \text{ molecules} & & & \\
 \hline
 & \times & & & \times & \\
 \text{ml cells} \cdot \text{min} & & \text{mole} & & & \\
 \\
 \hline
 1 \text{ ml} & & 1 \mu\text{l} & = & 1.86 \times 10^7 & \\
 & \times & & & & \text{molecules/cell min} \\
 \\
 1000 \mu\text{l} & & 1.2 \times 10^6 & & & \\
 & & \text{packed cells} & & &
 \end{array}$$

ATP hydrolysis calculations using original research data was as follows:

$$\begin{array}{ccccccccc}
 & \text{1st Assay} & \text{2nd Assay} & & & & & & \\
 & \text{moles} & \text{moles} & 1 \text{ liter} & \text{molecules} & \text{V} & & \text{1} & \\
 \text{Hydrolysis} = & \hline & \\
 \text{Rate} & \text{liter} & \text{liter} & 10^6 \mu\text{l} & \text{mole} & \text{N} & \text{min} & & \\
 & & & & & \text{cells} & & &
 \end{array}$$

= molecules/cell·min

where assays measure moles/liter of ATP,
 V= volume of test sample,
 N= number of cells in test sample with volume V.

APPENDIX III

ATP FLUX MOLECULES/ CM ² . SECOND)	2ND ASSAY			1ST ASSAY		
	MOLES LITER	LITER 10 ⁶ UL	• 200 UL	MOLES LITER	LITER 10 ⁶ UL	• 200 UL

APPENDIX IV

```

10: "A":PAUSE "ATP FLUX"
11:N=6
20:DATA 28.3E-9,49.9E-9
      ,0,72,0,99400
21:DATA
40:DIM T(N)
50:FOR R=1 TO N
60:READ T(R)
70:NEXT R
80:D=T(1)*0.0002
90:E=T(2)*0.0002
99:IF T(3)=0 THEN GOTO
      110
100:F=T(3)*0.0002
110:G=(E-D)*6.02E23/(T(4)
      )*T(6))
120:IF G<0 THEN LET G=0
129:IF T(5)=0 THEN GOTO
      150
130:H=(F-E)*6.02E23/(T(5)
      )*T(6))
140:IF H<0 THEN LET H=0
150:I=G*1E8/(422*60)
160:IF I<0 THEN LET I=0
170:J=(E-D)*1E8/(T(4)*T(
      6)*422*60)
180:IF J<0 THEN LET J=0
189:IF H=0 THEN GOTO 222
190:K=H*1E8/(422*60)
200:IF K<0 THEN LET K=0
209:IF T(5)=0 THEN GOTO
      220
210:L=(F-F)*1E8/(T(5)*T(
      6)*422*60)
220:IF I<0 THEN LET I=0
222:W=T(1)/T(6)
223:Y=T(2)/T(6)
224:IF H=0 THEN GOTO 228
225:Z=T(3)/T(6)
228:USING "###.###^"
229:PRINT = I,PRINT
230:PRINT "W= ";W
240:PRINT "Y= ";Y
250:PRINT "Z= ";Z
260:PRINT "G= ";G
270:PRINT "I= ";I
280:PRINT "J= ";J
290:PRINT "H= ";H
300:PRINT "K= ";K
310:PRINT "L= ";L
320:END

```

Program to Calculate ATP Fluxes

Input Data: 1) first assay
 2) second assay
 3) third assay
 4) minutes between 1) & 2)
 5) minutes between 2) & 3)
 6) total cells in 200 μ l

W= first assay in M/cell
 Y= second assay in M/cell
 Z= third assay in M/cell

G= first flux in molecules/cell·min
 I= first flux in molecules/cm²·sec
 J= first flux in moles/cm²·sec

H= second flux in molecules/cell·min
 K= second flux in molecules/cm²·sec
 L= second flux in moles/cm²·sec

APPENDIX V

Rate of ATP Hydrolysis by Frog Myocardium

	Assay	C.O.	Ventricle Wet Weight
Rate =	$\frac{\text{moles}}{\text{liter}} \times \frac{6.02 \times 10^{23}}{\text{mole}} \times \frac{\text{ml}}{\text{min}} \times \frac{1000 \text{ mg}}{1 \text{ gm}} \times \frac{1 \text{ liter}}{1000 \text{ ml}} \times \frac{1}{\text{Wt}}$		
			(mg)
=			$\text{molecules/gm} \cdot \text{min}$

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Vita Auctoris

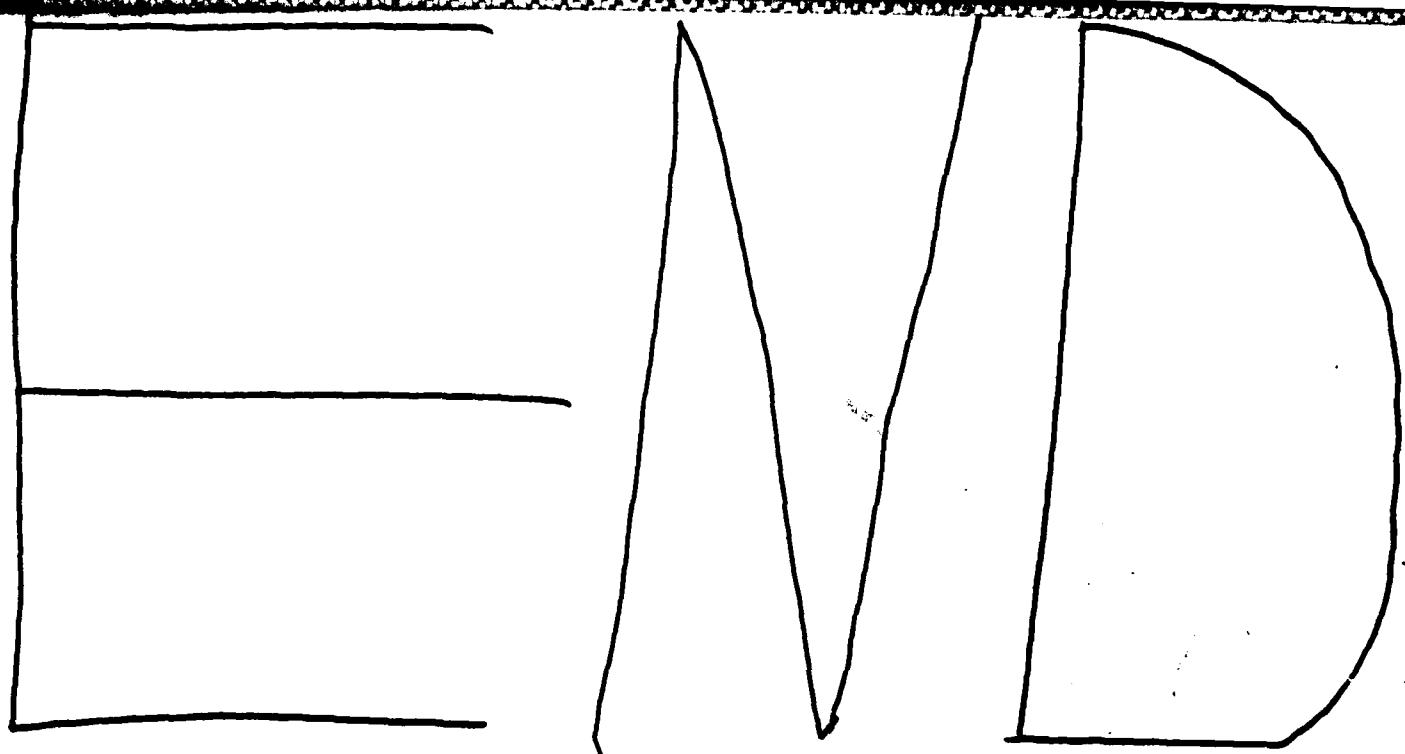
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